DOCTOR OF PHILOSOPHY

Investigating the regulation of the Notch pathway ligand Delta-like 1 in the vertebrate segmentation clock

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Abbreviations

ADAM	A Disintegrin And Metalloproteinase
AGO	Argonaut
AVS	Abnormal Vertebral Segmentation
AP	anterior-posterior
APC	adenomatous polyposis coli
bHLH	beta Helix-Loop-Helix
BMP	bone morphogenic protein
CBF	CCAAT-binding factor
CDC42	cell division cycle 42 homologue
CDK	cyclin dependent kinase
CK	casein kinase
C-myc	Cellular myc
CNH	chordoneural hinge
Co-A	Co-Activator
Co-R	Co-Repressor
CSL	CBF1/RBPjκ/Su(H)/Lag-1DNA binding protein
Cys	Cysteine
Dll	Delta-like
DML	Dorsal Medial Lips
Dvl	dishevelled
ECM	Extracellular Matrix

EGF	epidermal growth factor
EMT	Epithelial-Mesenchymal Transition
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
FGFR	FGF receptor
FN	Fibronectin
FRS2	Fibroblast growth factor receptor substrate 2
Fz	Frizzled
GBP	GSK3 binding protein
GEF	Guanine nucleotide exchange factor
Grb2	Growth Factor Receptor-Bound 2
GSK	Glycogen synthase kinase
HES	Hairy/enhancer of split
HH	Hamburger-Hamilton
Hox	Homeotic
Ig	Immunoglobulin
IHC	Immunohistochemistry
IP	Immunoprecipitation
LEF	Lymphoid enhancer-binding factor
Lfng	Lunatic Fringe
LPM	Lateral Plate Mesoderm
Mam	Mastermind
Mesp	Mesoderm posterior
MET	Mesenchymal-epithelial transition

MiRISC	MiRNA-induced silencing complex
MiRNA	MicroRNA
MO	Morpholino
Myf5	Myogenic Factor 5
MyoD	Myogenic differentiation 1
NICD	Notch intracellular domain
NEXT	Notch extracellular truncation
Nrarp	Notch-regulated ankyrin repeat protein
NSB	Node-Streak Border
NT	Neutrophin3
P.body	Processing body
Pax	Paired Box
pERK	Phospho-ERK
PI3K	Phosphoinositide 3-kinase
PSM	Presomitic Mesoderm
RA	Retinoic acid
Raldh	Retinaldehyde dehydrogenase
RBPjK	Recombination signal Binding Protein for immunoglobulin Kappa J
SCD	Spondylocostal Dysostosis
sFRP	Sluble Frizzled Receptor Protein
SH	Src Homology
SHH	Sonic Hedgehog
SOS	son-of-sevenless
STD	Spondylothoracic Dysostosis

TACE	tumour necrosis factor alpha converting enzyme
TBX	T-Box
TCF	T-cell specific transcription factor
VLL	Ventral Medial Lip
vt	vestigial tail
WIF	Wnt inhibitory factor

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Signed declaration

I, the candidate, am the author of this thesis and have consulted all references cited. The work presented here is a record of my individual work, except where otherwise stated and this work has not previously been accepted for a higher degree.

Signed,

Robert A. Bone

Supervisor statement

The conditions of the relevant Ordinance and Regulations have been fulfilled.

Signed,

J. Kim Dale

Abstract

The process of segmentation in vertebrate species is crucial for the production of a rigid body structure, and when problems with this process occur it often results in malformations of the skeleton. During segmentation in developing vertebrate embryos, pairs of somites, precursors to the vertebrae and ribs, form periodically along the axis of the embryo. This process is regulated by a molecular oscillator that controls the periodic expression of "clock" genes in the presomitic mesoderm (PSM), the rod of mesenchyme in the tail end of the embryo from which somites form. These oscillating clock components are thought to be required for somitogenesis to occur. All clock genes identified so far belong to the Notch, Wnt and Fibroblast Growth Factor (FGF) pathways, and these three pathways are essential to somitogenesis. However, very little is known about how these pathways interact.

In this thesis, I firstly investigate how the transcription of mRNA for Notch ligand DLL1 is regulated in the PSM, in order to determine whether it may act as a cross-talking point between Notch and Wnt pathways. This work shows that the pre-spliced mRNA for *Dll1* cycles across the mouse PSM in the manner of a clock gene, out of synchrony with Notch target clock genes but in synchrony with Wnt target clock genes. When we used the γ-sectetase inhibitor LY-411575 to inhibit Notch signalling we saw no significant down regulation of *mDll1*, and when we down regulated Wnt signalling with the Axin2-stabilising drug XAV-939 we saw more significant, but not complete, down regulation of *mDll1*. However, when we inhibited both Notch and Wnt signalling pathways with these drugs, we saw *mDll1* expression disappear.

I also aim to ask the question of whether clock gene oscillations are required for the process of somitogenesis: for this I used the Hes7-/- and CatC-Lef1 mutant mouse lines to look at

how clock gene oscillations are affected in the PSM of mice with constitutive Notch activity and constitutive Wnt activity respectively. I also investigated the effects of Notch inactivation and halting of cyclic Notch activity in the chick PSM, which determines that the presence of dynamic Notch activity in the PSM is required for somitogenesis to occur.

Finally, I investigated the effect of the loss of microRNA (miRNA) activity, which mediates translational repression of mRNAs, in a conditional mutant mouse line in which this activity is lost. The skeletal structures of mice with this deficiency show severe malformations of the spine and ribs, and appear to be independent of clock gene activity.

Introduction

The process of segmentation is a universal feature of the body plan of all vertebrate species, as well as many invertebrate species. This is most clearly seen in the form of the vertebrate axial skeleton, which is composed of segmented structures in the form of ribs and articulating vertebrae. In vertebrate species the process of segmentation begins with the sequential formation of structures called somites, which later develop into the ribs and vertebrae. The process of somitogenesis initiates at an early phase of vertebrate embryonic development, and interference with this process can lead to serious segmental defects and associated pathologies, such as Spondylocostal dysostosis (SCD). SCD is a group of conditions which are characterised by abnormal development of vertebrae and ribs, where the vertebrae are misshapen and fused together. To gain a deeper understanding of why, when and where these defects occur and how they might be treated, it is therefore important that the molecular mechanisms behind the segmentation process are understood by studying somitogenesis in animal models.

1. Human Pathologies

Abnormal vertebral segmentation (AVS) is a conglomerate of various spinal disorders in humans. The true prevalence of AVS in humans is hard to determine, as not everyone with AVS is diagnosed (Turnpenny et al., 2007). Because of the large variety of phenotypes associated with this condition, the number of cases in which the phenotype can be identified (i.e. by X-ray or MRI imaging) is small, and subsequently the aetiology of AVS remains largely unknown. One clear contributing factor is that when somitogenesis (the early developmental process which is the first step towards building a segmented body axis) is disrupted by either environmental or genetic factors, skeletal defects arise. Non-syndromic forms of AVS, i.e. where the pathologies are restricted to specific regions of the spine, are commonly referred to as types of Spondylocostal Dysostosis (SCD Types I-IV) (Turnpenny et al., 2007). A common feature of SCD is the abnormal side-to-side curvature of the spine, known as scoliosis, which is caused by the malformation of the vertebrae. Due to these malformations, affected individuals have short necks and midsections and as a result have short bodies, but have normal arms and legs, resulting in short-trunk dwarfism. Although this condition is rare, it is unknown how many individuals are affected by this condition. Due to the shortening of the thorax, respiratory functions in newly born babies may be compromised (although by the age of two, lung function can improve sufficiently, life-threatening complications such as pulmonary hypertension can still arise in children with restricted lung capacity).

Spondylothoracic Dysostosis (STD) is also a form of AVS, and is characterised by a malformation of the ribs, causing them to fan out in a ‘crab-like’ fashion, as well as a severely shortened trunk. Infant mortality for these conditions is thought to be a roughly

50% due to restricted breathing ability, although an early diagnosis and care improves the prognosis (Turnpenny et al., 2007).

1.1 Notch signalling and SCD

As mentioned above, the aetiology of most AVS Syndromes is unknown. However one signalling pathway that has been associated with several human disorders such as SCD is the Notch signalling pathway (Figure A) (Turnpenny et al., 2007). As discussed in detail below this pathway is required for somitogenesis in all vertebrate animal models studied to date (including fish, frog, chick and mouse). Notch signalling enables short-term communication between cells because both the receptor and the ligand are membrane-bound proteins, and is able to either promote or repress various processes, such cell proliferation, cell death and acquisition of specific cell fates depending on the context (Kopan & Ilagan, 2009). It is an unusual signalling pathway in some respects, as it relies on the ability of a ligand to bring about receptor proteolysis which results in the release of an active Notch fragment that then acts as a transcriptional activator in the nucleus.

The gene for the Notch receptor was first characterised in *Drosophila melanogaster*, and was shown to encode a 300 kDa single-pass receptor (Wharton et al., 1985): the name was derived from the phenotype observed in flies that were haploinsufficient (i.e. had a partial loss of function) for the gene, and had 'notches' in the wing margins (Mohr, 1919). Although there is only one Notch receptor gene in *Drosophila*, there are four Notch receptors (mNotch1-4) in mammals (Bray, 2006).

Other key components of the core Notch pathway are the ligands. In Drosophila the two single-pass transmembrane proteins, Delta and Serrate, function as partially redundant ligands (Greenwald, 1998); in vertebrates these are Delta-like (Dll1, Dll3, and Dll4) and Jagged (Jagged1 and Jagged2) respectively (Gridley, 1997).

The newly translated Notch receptor protein is glycosylated in the Golgi by O-fut and Rumi, which are required to produce a fully functional receptor. The mature receptor is then produced following proteolytic cleavage in the Golgi by PC5/furin at site 1 (S1). The receptor is then targeted to the cell surface as a heterodimer, held together by non-covalent interactions. The Notch receptor is activated by binding to a ligand on a neighbouring cell. In cells expressing the glycosyltransferase Fringe, the O-fucose is extended by Fringe enzymatic activity in the Golgi prior to the receptor bring targeted to the cell surface, thereby altering the ability of specific ligands to bind to Notch. Endocytosis and membrane trafficking regulate receptor and ligand availability at the cell surface. Ligand endocytosis generates the mechanical force to promote a conformational change in the bound Notch receptor. This conformational change exposes site 2 (S2) in Notch for cleavage by A Disintegrin And Metalloproteinase (ADAM), and juxtamembrane Notch cleavage at S2 generates the Notch extracellular truncation (NEXT) fragment, a substrate for the γ-secretase complex. γ-secretase cleaves the Notch transmembrane domain in NEXT progressively from S3 to S4 to release the Notch intracellular domain (NICD) and the Nβ peptide. γ-secretase cleavage can also occur at the endosome, but cleavage at the membrane generates a more stable NICD. NICD then enters the nucleus and associates with CSL (CBF1/RBPjκ/Su(H)/Lag-1), a DNA-binding protein (the only transcription factor in the core Notch signalling pathway). In the absence of NICD, CSL may associate with ubiquitous co-repressor proteins (Co-R) and

histone deacetylases (HDACs) to repress target gene transcription. Upon NICD binding, these transcriptional repressors are displaced, thought to be due to allosteric changes that occur in CSL. Mastermind-1 (Mam1), a transcriptional co-activator (Co-A), then recognises the NICD/CSL interface, and this complex recruits additional Co-As to activate Notch target gene transcription.

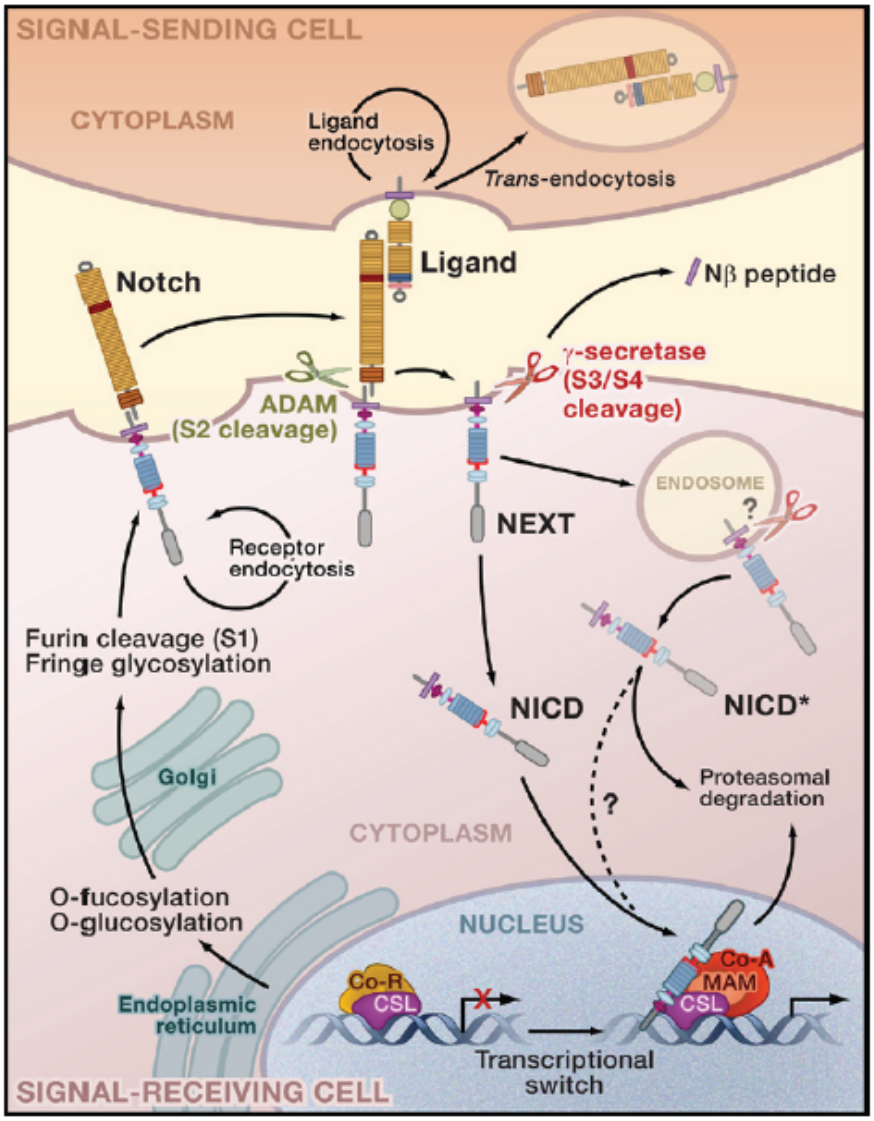


Figure A: The Canonical Notch signalling pathway

Following its translation, the Notch receptor protein is glycosylated in the Golgi. The receptor is then proteolytically cleaved to form a hetero-oligomer, and is targeted to the cell surface. The Notch receptor binds to a Delta ligand, which induces ADAM metalloproteases to cleave the Notch receptor to generate NEXT. γ-secretase then cleaves NEXT to generate NICD. NICD then translocates to the nucleus, where it de-represses the CSL DNA-binding protein, along with other co-activators such as Mastermind (Mam) (Figure taken from Kopan & Ilagan, 2009).

There have been numerous studies which have shown that mutations of several genes that are involved with the Notch signalling pathway cause several of these autosomal recessive segmentation/SCD pathologies. In particular, these studies have shown that DLL3, MESP2, LFNG and HES7 mutations have all caused severe segmentation defects (Figure B). The human pathologies caused by mutations in these components will be discussed next. The mode of action and role in somitogenesis of these different factors will be discussed below in Section 5, where I discuss the molecular regulation of somitogenesis in the embryo.

1.2 DLL3

The *Dll3* gene encodes a ligand for Notch signalling, and is thought to have a key role in somitogenesis (see below in Section 5). Homozygous mutations in *Dll3* in humans causes SCD Type I, where all vertebrae are affected and lose their regular shape: thoracic vertebrae are most heavily affected, with the rib being misaligned (Turnpenny et al., 2007). There are 24 known specific mutations in Dll3 which have been identified in patients with SCD, and all but one (which affects the transmembrane region) occur in the region of exons 4-10, and are thought to comprise nonsense, missense, frameshift, splicing and frame insertion mutations that result in truncation of the extracellular domain of the protein (Bonafé et al., 2003; Turnpenny et al., 2003). *Dll3* mutations have not been observed in other phenotypes such as multiple vertebral segmentation defects (MVSD), which means that there is very little variance in phenotypes due to mutated *Dll3* which significantly improves the application of genetic testing in a clinical context. *Dll3* was the first Notch pathway gene identified in relation to human segmental disorders, and led to a breakthrough in the

understanding of how these disorders arise, as well as leading to the identification of other genes which are involved in SCD cases.

1.3 MESP2

Mesoderm posterior 2 (MESP2) is a basic helix-loop-helix (bHLH) transcription factor that acts downstream of Notch and is crucial for somite boundary formation (see below in Section 11) for more detailed explanation of its role in boundary formation) (Saga, 2007)). *Mesp2*-/- mice develop somites which are completely caudalised, causing segmental defects to occur. The pups die roughly 20 minutes after birth with all but a few caudal vertebrae affected and short trunks, similar to the *Dll3*-/- mice (Saga et al., 1997; Turnpenny et al., 2007). However, the mutant protein in humans retains its bHLH region and can still bind to DNA, unlike the mouse null *Mesp2* allele (Whittock et al., 2004). In humans with a homozygous 4bp duplication mutation in exon 1 of the *Mesp2* gene, the thoracic vertebrae are severely affected, with the lumbar vertebrae only being mildly affected. This condition is known as SCD Type II, and there is only one known mutation which causes this phenotype (Turnpenny et al., 2007; Whittock et al., 2004).

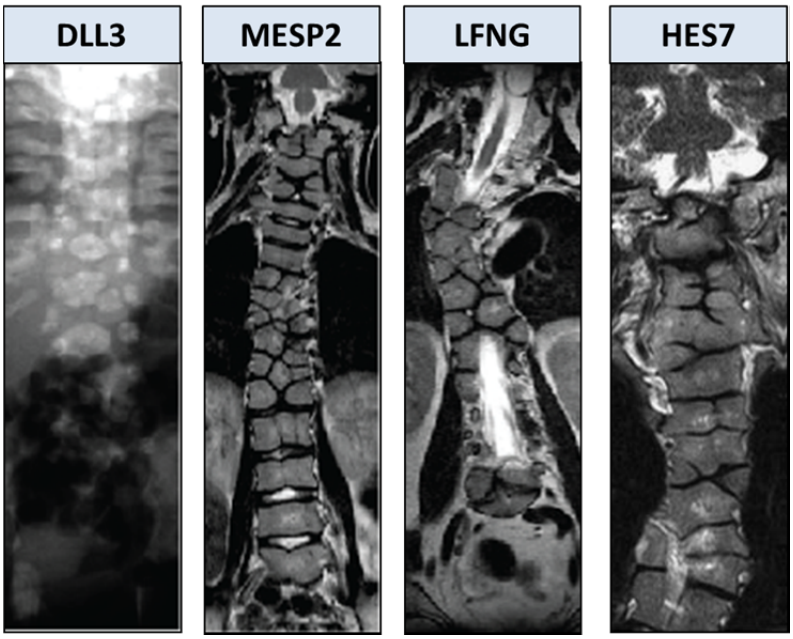
1.4 LFNG

Lunatic fringe (LFNG) is a target of Notch signalling and is also a glycosyltransferase which modifies the ability of the Notch receptor to interact with its ligands. *Lfng*-/- mice form somites which are irregular in size and shape, and most die shortly after birth, which is thought to be because of respiratory failure due to malformed rib cages (N. Zhang & Gridley,

1998). The phenotype in human cases, which involves the shortening of the neck and trunk due to severe rib abnormalities and multiple hemivertebrae, is thought to be caused by a homozygous missense mutation in exon 3 of the *lfng* gene (Sparrow et al., 2006), and is designated SCD type III.

1.5 HES7

Hairy and Enhancer of Split 7 (HES7) is a bHLH transcription factor which is both a target and a negative regulator of the Notch signalling pathway. In *Hes7*-/- mice, there are severe segmentation defects, with abnormally formed ribs and vertebrae which are significantly short, and are fused and bifurcated (Bessho et al., 2001). In humans a missense mutation in the DNA-binding domain of HES7 which inhibits binding to DNA or formation of a heterodimer (Sparrow et al., 2008), causes the offspring to show a shortened spine, with all of the vertebrae showing multiple segmentation defects which were most pronounced in the thoracic region. This was diagnosed as SCD Type IV, as it was morphologically distinct from SCD Types I-III (Sparrow et al., 2008).



Gene	Function	Chromosomal locus	Condition	Phenotype
DLL3	Notch ligand	19q13	SCD-Type1	Abnormal vertebral segmentation throughout the entire spine, with all vertebrae losing normal form and shape.
MESP2	bHLH transcription factor	15p26	SCD-Type2	Thoracic vertebrae severely affected, lumbar vertebrae only mildly affected.
LFNG	Notch receptor modifier	7p22	SCD-Type3	Short neck, short trunk. Spinal MRI shows severe rib abnormalities and multiple hemivertebrae.
HES7	bHLH transcription repressor	17p13	SCD-Type4	Shortening of the spine, multiple segmentation defects mainly in the thoracic region.

Figure B: Human Pathologies associated with Notch pathway mutations

Mutations in four genes which are members of the Notch signalling pathway, and are crucial to early patterning in the PSM, cause defects in somitogenesis and subsequently lead to muscular deformities such as SCD. These defects can be seen in MRI scans, where gene mutations result in irregularly sized and fused vertebrae, and each gene mutation results in a distinct SCD type (Adapted from Maroto et al., 2012).

2. Segmentation in Drosophila and other arthropods

The work carried out in *Drosophila* has been the basis for our understanding of the segmentation process in embryogenesis. However, the mechanism of segmentation in *Drosophila* is fairly atypical when compared to other arthropods. In *Drosophila*, all the segments are formed simultaneously, as a segmentation gene cascade subdivides the embryo into smaller units. This process of segmentation is known as the 'long germ' mode of development. This, however, differs from the segmentation of most other arthropods, in which segments are generated sequentially from a posterior growth zone (termed the 'short germ' mode of development), which indicates that the simultaneous formation of segments adopted by *Drosophila* is a derivation of the ancestral short germ mode (Damen, 2007). In *Drosophila*, the syncytial blastoderm provides an ideal environment for the establishment of morphogen gradients, as the diffusion of transcription factors to their required locations is relatively easy. However, in other arthropods, which are multicellular at the point of segmentation, this becomes impractical, and so segment formation must be sequential. This poses the question: to what extent does the *Drosophila* model of segmentation represent the model of segmentation in other invertebrate species? Research carried out in the last decade has shown that for example: in the spider segmentation is governed by activity of Notch and Delta genes (which have no role in *Drosophila* segmentation), as determined through RNA interference (RNAi) experiments (Stollewerk et al., 2003), indicating that this method of segmentation is closer to the vertebrate somitogenesis model rather than *Drosophila* segmentation. The role of Delta-Notch signalling was also identified in the centipede and the insect Tribolium (McGregor et al., 2009), but with no true explanation of how Delta-Notch signalling regulated segmentation. The first functional insight into this role

was identified in the cockroach model, where Delta-Notch signalling was shown to establish the growth zone, and was responsible for subsequent segment generation from this tissue. This was shown through disruption of Notch/Delta signalling, which resulted in missing abdominal segments within the cockroach embryo; a consequence of a disrupted posterior growth zone (Peuyo et al. 2008; McGregor et al. 2009). These findings shown that short germ band formation, which is regulated by Delta-Notch signalling, is likely to be the key ancestral mechanism of segmentation, with *Drosophila* long germ band formation being an aberration from this mechanism.

3. Gastrulation and generation of the Presomitic Mesoderm

Gastrulation is the stage in embryonic development where the three germ layers are formed: the ectoderm, mesoderm and endoderm; that will eventually form all the tissues of the embryo proper. The site of gastrulation in avian, reptilian and mammalian embryos is a structure known as the primitive streak. In the chick embryo, the primitive streak cells arise locally in the posterior marginal region, and no other cells in the embryo are involved in its creation (Lawson & Schoenwolf, 2001). The primitive streak is first visible as a thickening of the epiblast at the posterior marginal region, anterior to Koller's sickle, and this is due to the increased height of the layer of cells forming the centre of the primitive streak. As the streak progresses, the increased length is directly proportional to the decrease in width, which indicates that the cell population within the streak is unchanging, and these cells which formed the initial streak are migrating anteriorly. During streak elongation a primitive groove forms within the streak, allowing the passage of migrating cells into the underlying blastocoel. At the anterior end of the primitive streak the organiser (Hensen's node in the chick; node in the mouse), is formed by a thickening of cells which also contains a funnel shaped groove, and allows the passage of migrating cells into the blastocoel, which is a fluid-filled cavity in the middle of the blastocyst and is crucial to allowing cell movements during gastrulation.

In mouse and chick, the first cells to ingress through the primitive streak into the blastocoel are epiblast cells, and as they ingress they undergo an epithelial-to-mesenchymal transition (EMT). The EMT involves a dramatic change in cell morphology and behaviour which allows them to break down the basal lamina, internalise, and migrate towards their destination. As these cells migrate inwardly and enter the blastocoel they separate into two layers: the first

is a deep layer of cells which displaces hypoblast cells on either side of the streak and eventually give rise to all the endodermal organs of the body, as well as some extra-embryonic tissue; the second is a loose layer of cells between the epiblast and the endoderm which will form all the mesodermal tissues.

The epiblast cells which do not ingress will remain epiblast cells and will eventually form ectodermal derivatives (Acloque et al., 2009; Harrisson et al., 1991). Thus, the primitive streak defines the embryonic axis: cells which ingress through the Hensen's node and the anterior streak enter the blastocoel and form the mesodermally derived tissues which lie along the midline of the embryo, such as head mesoderm, notochord and foregut, while cells which ingress more posteriorly form the majority of endodermal and more laterally located mesodermal tissues (Schoenwolf et al., 1992). Like chick epiblast cells, the mammalian mesoderm progenitor cells also ingress through the primitive streak (Burdsal et al., 1993).

As mesodermal ingression continues, the primitive streak and Hensen's node begin to regress, and leaves in its wake the dorsal axis of the embryo and notochord. Formation and maturation of the endoderm, mesoderm and neural tube occur in synchrony in an anterior-to-posterior direction, and either side of the neural tube are thick bands of mesodermal cells which will eventually form the somites called the presomitic mesoderm (PSM). Fate mapping studies in the mouse embryo have demonstrated that this ectoderm-to-mesoderm transition continue late into axis elongation, generating the cells that pass into the PSM to form somites (Cambray & Wilson, 2002; Wilson & Beddington, 1996).

Fate map studies of the primitive streak in the early stage chick embryo have shown that the contribution of specific regions of the streak contribute to medial and lateral halves of the

somites (Psychoyos & Stern, 1996). Prior to these studies it was also shown that progenitors of the medial halves of somites reside in the Hensen's node before they ingress into the blastocoel, while the location of the progenitors of the lateral halves of somites are found more posteriorly in the streak (Selleck & Stern, 1991). This correlated with another study which stated that the two halves of dermamyotome have different fates, as they contribute to different muscles (Ordahl & Le Douarin, 1992). However, this differs with other findings which suggested that there was no segregation of precursors at Hamburger Hamilton (HH) stage 4+ (Schoenwolf et al., 1992). Since these findings it has been shown that although there is a segregation of precursors, there is a higher degree of overlap than was previously thought. In one study, it was shown in the chick embryo that the Hensen's node (medial, intermediate or lateral compartments) is the only region in which cells contribute solely to the medial part of somites, and that the lateral somite precursors reside between positions 57% and 91% of the streak, whereas some cells which contribute to the medial part of somites can be located as far posteriorly as position 73%, showing that there was a significant degree of overlap of these progenitors. Therefore, the medial precursors migrate through the Hensen's node or anterior primitive streak to reach the medial PSM, whereas the lateral precursors migrate more posteriorly to reach the lateral half of the PSM (Psychoyos & Stern, 1996). In the mouse embryo, it has been demonstrated that somites are derived from a much higher proportion of the primitive streak than in the chick (80% rather than 20%) (Cambray & Wilson, 2007).

In the mouse embryo, the process of axis elongation lasts for 6 days and results in the formation of 60 somites. Previous clonal lineage studies have shown that long term axial progenitors are present in the primitive streak that give rise to axial structures over long

rostro-caudal distances, and can be called “stem cell-like” in character. Transplantation studies have shown that self-renewing cells exist in the chordoneural hinge (CNH) of the mouse tail bud (Cambray & Wilson, 2002); in these experiments, CNH cells were transplanted from E10.5-12.5 mice to the primitive streak region of E8.5 embryos and left to culture for 48 hours. Following this culture period the transplanted cells showed contribution to rostral somites, the notochord and the neural tube, showing that progenitor cells of the caudal axis retain the capacity to contribute to rostral tissues, and therefore are ‘stem cell-like’ in character.

Subsequent studies using homotopic grafting techniques showed that the cells which are derived from the node and primitive streak mainly contribute to the mesoderm (in another study using chick embryos, grafting experiments have confirmed that Hensen’s node cells also contribute to the floor plate of the neural tube (Gray & Dale, 2010)), whereas node-streak border (NSB) cells also contribute to the neuroectoderm and CNH cells. Ectodermal cells lateral to the primitive streak at E8.5 also contribute to the somites and CNH (Cambray & Wilson, 2007).

Retrospective clonal analysis in the mouse embryo with the LacZ system have revealed that the murine primitive streak has 100-150 somitic stem-like cells, which generate progenitors of somites all along the mouse axis (Nicolas et al., 1996).

Despite evidence that cells in the primitive streak contribute to tissues over a long distance, they are not true stem cells because their gene expression changes over time, so they do not self-renew to generate entirely identical daughters (Cambray & Wilson, 2007; Iimura & Pourquié, 2006). McGrew et al. conducted a series of grafting experiments using a GFP-transgenic chicken line to show that when GFP positive CNH cells are grafted to the CNH of a

non-GFP expressing chick embryo (at the same stage of development) the GFP positive axial progenitors in the CNH, which normally produce neural and mesodermal descendants, change their fates according to which region of the embryo they are grafted to, as well as losing some of their potency by failing to be retained in the tail bud. This led the authors to conclude that the fates of long term axial progenitors (LTAPs) in the CNH are undetermined, and rely on a CNH environment to develop into mesodermal and neural descendants (McGrew et al., 2008).

3.1 Hox genes and gastrulation

Depending on the location of the somite, it will differentiate into a vertebra with a distinct anterior-posterior identity. This regionalisation of vertebrae along the anterior-posterior axis of the embryo is regulated by a group of transcription factors called Homeobox-containing (*Hox*) genes, which specify the anterior-posterior identity at the onset of gastrulation. The *Hox* genes are homologous to *Hom-C* (Homeotic selector genes) in *Drosophila*. In vertebrates there are four clusters of *Hox* genes located on four different chromosomes: *Hoxa* to *Hoxd*; which are thought to have arisen by chromosome duplications during the course of evolution (McGinnis & Krumlauf, 1992). *Hox* genes within each cluster are numbered 1 to 13, proceeding in an anterior-to-posterior direction. There is thought to be a large degree of conservation between flies and mammals.

Equivalent genes in each complex (e.g. *Hoxa1, Hoxb1*) are called a 'paralogous group', and genes from paralogous group 1 are expressed from the anterior border of the hindbrain to the tip of the tail. The genes from the paralogous group 2 are expressed throughout the

spinal cord, but their anterior expression boundary is two segments caudal to the paralogous group 1 boundary (Keynes & Lumsden, 1990; Wilkinson, et al., 1989).

In each cluster, the genes are arranged on the chromosome in order of expression: in mammals and short germ band insects, *Hox* genes located more 3' within the cluster are expressed in more anterior regions of the axis and at an earlier stage in development, then more 5' located *Hox* genes are expressed later in more posterior domains of the embryo. These aspects of the expression profile are referred to as spatial and 'temporal colinearity' (see Figure L) (Kmita & Duboule, 2003). 'Spatial colinearity' is the relationship between the order of *Hox* genes on their chromosome and the anterior-posterior region of expression (Kmita & Duboule, 2003). Nested expression of specific *Hox* genes along the anterior-posterior axis, initiated in the primitive streak, gives each vertebral precursor a unique combination of *Hox* genes that controls their axial identity (Kessel & Gruss, 1991; Wellik & Capecchi, 2003).

An elegant study in the chick embryo demonstrated that at a defined time point during gastrulation, the ingression of epiblast cells into the primitive streak is controlled by 3' *Hox* genes expressed by these cells. When the next more 5'-paralogous *Hox* gene becomes activated in a subpopulation of these cells, their ingression is slightly delayed, and expression of this *Hox* gene is spread rapidly throughout the other epiblast cells so that they acquire the same migratory properties. When the next more 5'-paralogous *Hox* gene is expressed in a subpopulation of the remaining epiblast cells, the same pattern is re-established until the next *Hox* gene is expressed, and so on. This ordered ingression of epiblast cells into the primitive streak is believed to regulate the establishment of the

nested *Hox* expression domains within the mesoderm, i.e. spatial colinearity (Iimura & Pourquié, 2006).

It was also shown in the chick embryo that CNH and dorsal posterior tail bud cells express Hox genes which are specific to their anterior-posterior level, but if these cell populations are grafted to a host embryo of a younger developmental stage, their expression profile changes to express the *Hox* genes of their surrounding environment. This indicates that the anterior-posterior identity of the cells in the tail bud is plastic, and will become specified later, prior to the formation of somites in the PSM (McGrew et al., 2008).

The precise temporal activation and sequential expression of *Hox* transcription is crucial for establishing regional identity, for example a Hoxc8 mutation in mouse which causes a transient delay in initial transcription of this *Hox* gene phenocopies the *Hoxc8*-/- mouse, thus affecting skeletal patterning(Juan & Ruddle, 2003).

4. Mathematical Models of Somitogenesis

Prior to the molecular era, somitogenesis had already been the focus of intense study for many decades. One aspect of this process which received a huge amount of attention was the progressive periodicity of the process. Numerous theoretical models were proposed to explain how this periodicity might come about and be regulated.

4.1 Clock and Wavefront model

The Clock and Wavefront model was derived from the Positional Information (PI) theory (Wolpert, 1994), which states that the PI variable of each somitic cell is a function of developmental time. At some critical point in development, the PI variable undergoes a sudden change, and since development proceeds in a rostro-caudal fashion, this change can be interpreted as a 'wave' of maturation. This acts in conjuncture with an intrinsic "clock" in the PSM cells, which regulates the response of phase-linked cells to react to this sudden change, i.e. when cells have passed a competence threshold they reach a certain point in their oscillation cycle which allows them to undergo segmentation (Cooke & Zeeman, 1976). The first experimental evidence to support this theory came when amphibian embryos were subjected to a 'heat shock' pulse, and then allowed to continue development. Segmentation in these embryos was disrupted in somites caudal to the pair forming at the time of heat shock, indicating the existence of susceptible phase-linked cells in the PSM (Cooke, 1978; Elsdale, Pearson, & Whitehead, 1976). However, this did not provide a clue as to how this oscillator works.

4.2 Meinhardt's model

Meinhardt's model provides an alternative explanation to somite formation to that provided by the Clock and Wavefront model. Like the clock and wavefront model, it proposes the existence of a molecular oscillator in PSM cells. However, whereas the Clock and Wavefront model explains the global regulation of somite number against embryo size, Meinhardt accounts for establishment of anterior-posterior polarity in somites, based on reaction-diffusion mechanisms. This model proposes that anterior-posterior polarity is initially set up in cells of the PSM prior to segmentation. The oscillatory mechanism in PSM cells producing alternating states, named anterior (A) and posterior (P), and the time taken for one oscillation to occur is the same amount of time taken for a pair of somites to form. At the anterior limit of the PSM, the cell states become fixed to 'A' or 'P' states, and cells cannot mix. A third state exists for the formation of somitic borders, formed between posterior cells of one somite and the anterior cells of another (Meinhardt, 1986). Again however, this model did not provide a clue as to how this oscillator works at the molecular level.

4.3 Cell Cycle Model

Claudio Stern and colleagues put forward the theory that the PSM clock is regulated by the cell cycle. This model was borne from an experiment in which chick embryos were subjected to a heat shock pulse, resulting in segmentation abnormalities which were repeated at 5-7 somite intervals up to three times. This correlates with the same time period as the cell cycle in the PSM (10 hours), since in the chick embryo segments form roughly every 90 minutes). The model postulates that heat-shock affected cells are grouped together and are

destined to populate a particular somite, and are in a specific 'heat-sensitive' phase of the cell cycle. Interestingly the vertebral levels of the axis: occipital, cervical, thoracic, lumbar, and sacral, are all comprised of 5-7 vertebrae. This indicates that cell cycle completion in these cells could be responsible for transitioning between these regional levels.

4.4 Einbahnstrasse Model

This model, proposed by Denis Duboule, was adapted from the cell cycle model with particular reference to the *Hox* genes, which was hypothesised after observing the proximo-distal outgrowth of the limb). The activation of *Hox* gene expression from the anterior to posterior regions of the axis is a function of cell proliferation. Only the cells which remain in the proliferative zone will undergo enough cell divisions to activate more 5' *Hox* genes and thus develop a more 'posterior' identity. The mechanism arises by accumulation of transcriptional repressor proteins which bind with high affinity to the 5' region of the Hox complex. With successive cell divisions, however, these proteins are eventually titrated out, and the transcription of more 5' *Hox* genes can occur. This model fits well with the spatial and temporal control of *Hox* genes in the PSM, thus the PSM cells which are produced early in the streak will express 3' *Hox* genes and become more anterior in character, whereas PSM cells produced after successive cell divisions will express more 5' *Hox* genes and be located more posteriorly. This combination of *Hox* genes expressed in the cells later on specifies their vertebral character along the body axis.

5. The Segmentation Clock Oscillator

Of these models, the one that has received the most support from experimental and molecular evidence is the “Clock and Wavefront” model (Cooke & Zeeman, 1976) (reviewed Dale & Pourquié, 2000; Goldbeter et al., 2007).

The first molecular evidence for the existence of a molecular oscillator acting in the PSM to regulate the formation of somites was the expression analysis of the Notch signalling pathway target gene *cHairy1* which showed dynamic oscillatory expression at the mRNA level in the chick PSM (Palmeirim et al., 1997). This dynamic expression profile appears as a wave of transcription that sweeps up the PSM in a caudal-to-rostral fashion. The time taken for this wave of transcription to sweep the PSM is the time it takes for a somite pair to bud off the rostral PSM and subsequently, another wave of transcription appears again in the caudal PSM.

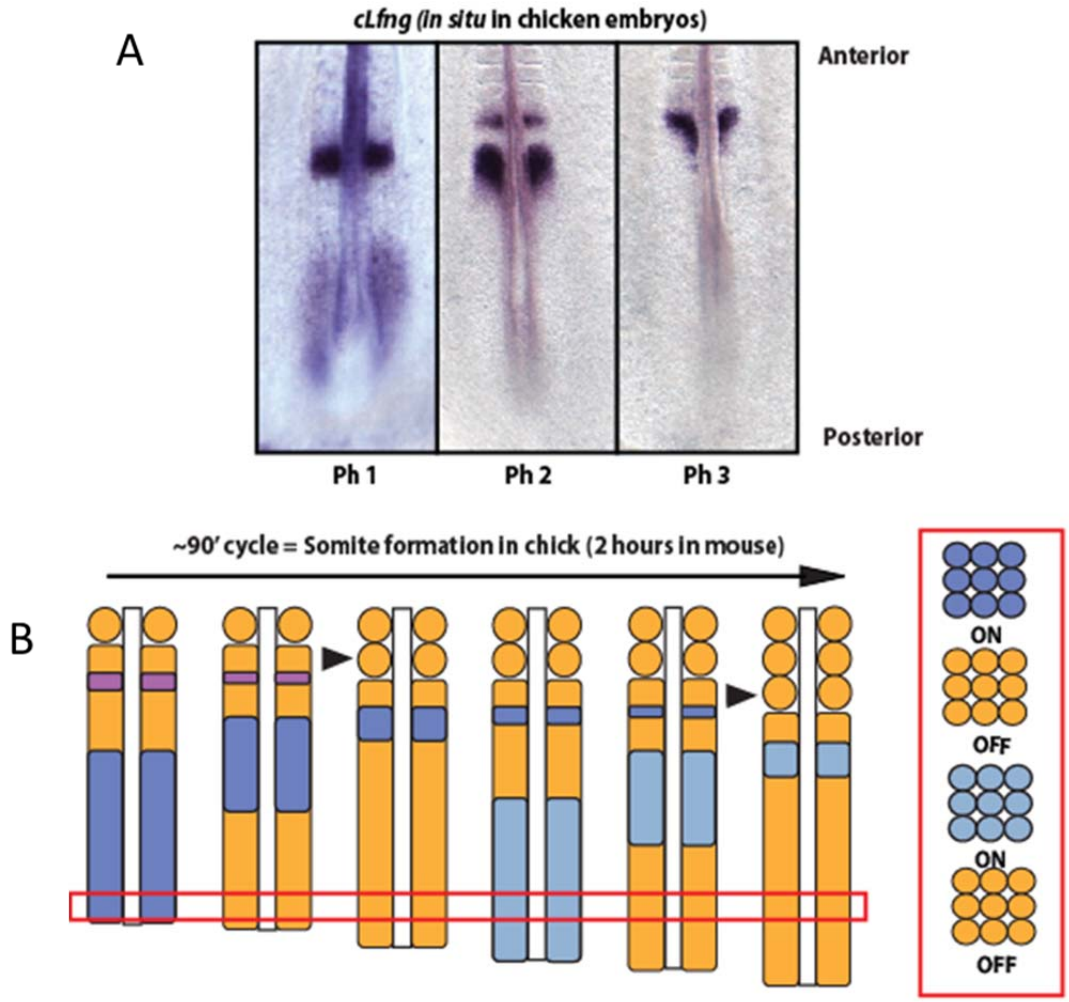


Figure C: The vertebrate segmentation clock

The process of somitogenesis is governed by a molecular oscillator or 'segmentation clock', which acts in the PSM cells to drive the dynamic and periodic expression of 'clock' genes across the PSM as evidenced by *cLfng* expression in the chick embryo (A). In this model, individual cells turn on and off gene expression in a synchronised fashion, giving rise to a wave of transcription which sweeps across the PSM in a rostral-to-caudal fashion with a periodicity to match somite formation (B). The period of oscillation varies between species: in chick, one cycle is 90 minutes; in mouse, one cycle lasts 2 hours.

Since this initial discovery, many other genes have been found to oscillate in this manner in the PSM of chick, mouse, frog, and zebrafish embryos, indicating that this oscillator is conserved among vertebrate species. The *Hes/Her/Hairy* bHLH Notch target genes show oscillatory expression in all of these vertebrate animal models. They encode transcriptional repressor proteins which have previously been shown to repress their own transcription (Bessho et al., 2001a; Jouve et al., 2000; Palmeirim et al., 1997). It is widely held that this type of negative feedback loop generates oscillatory Notch activity, which is important for somitogenesis. The first direct evidence for cyclic Notch activity was the identification of NICD by immunohistochemistry, which was shown to exhibit different phases of expression in the PSM similar in pattern to that of *cHairy1* mRNA expression (Huppert et al., 2005; Morimoto et al., 2005).

The concept that HES proteins regulate the oscillations of Notch activity relies on a short half-life of these repressor proteins. Indeed it was shown that this is the case for HES7 which has a half-life of 22 minutes in the mouse PSM. Moreover, the importance of this half-life to correct oscillatory clock gene expression was proven when Hes7 had a lysine-to-arginine point mutation introduced that extended the half-life of the protein to 30 minutes. This transformation resulted in the disruption of somite formation, which was caused by the abolition of cyclic Notch activity (Bessho et al., 2003). It has also been shown that Hes7-/- mice show an increase in transcriptional expression of *Hes7* and *Lfng*, showing that HES7 represses its own transcription, as well as the transcription of the other Notch target gene that encodes the Notch activity modifier LFNG (Bessho et al., 2003). In addition to *Hes7*, *Hes1*, *Hes5* and *Hey2* all show dynamic oscillatory patterns in the mouse PSM. However,

homozygous null mice for each of these genes have no somitic phenotype except those homozygous null for Hes7.

Lunatic fringe, the Notch-modifying glycosyltransferase enzyme, modifies the ability of the Notch receptor to bind to ligands (Moloney et al., 2000), and also oscillates in both mouse and chick PSM (Forsberg et al., 1998; McGrew, et al., 1998; Aulehla & Johnson, 1999). This dynamic expression is Notch dependent (Dale et al 2003). When overexpressed, in the chick PSM LFNG abolishes all cyclic gene expression, and somitogenesis is disrupted (Dale et al., 2003). It was thus proposed that LFNG acts to negatively regulate Notch-Delta interaction in the PSM and thus its own transcription, and therefore that of other Notch targets, thereby establishing a negative feedback loop. This repression is transient, since LFNG protein and mRNA is unstable, (Dale et al., 2003). However, an up regulation of *Lfng* gene expression in the mouse PSM does not abolish the oscillations of LFNG and HES7 at the protein level (Serth, Schuster-Gossler, Cordes, & Gossler, 2003) , which indicates that the *Lfng* negative feedback loop is insufficient to drive oscillations, at least in the mouse PSM, by itself.

Interestingly, the DLL-3 ligand (see Section 1.2) has been shown to be essential to dynamic gene expression in the mouse embryo: in *Dll3* -/- mice, the oscillations of *Hes1, Hes5* and *Lfng* are inhibited (Dunwoodie et al., 2002; Kusumi et al., 2004). The disruption of these cyclic genes in the PSM fits up with the subsequent skeletal defects observed in humans and mice (see Section 1.2), and indicates the important link between the segmentation clock and development of the axial skeleton.

Many Notch target genes are dynamic in the PSM, which indicates that Notch is a central component of the segmentation clock. To support this idea, mice lacking different components of this pathway exhibit severe segmentation defects (Rida et al., 2004). An

example of this is that in mice which have a lack of presenilin (Psen) proteins (key components of the γ-secretase complex), in which Notch activity is completely abolished, cyclic gene expression and segmentation does not occur at all. This phenotype can be recapitulated by culturing mice embryos in the presence of the small molecule γ-secretase inhibitor LY411575 (Ferjentsik et al., 2009).

Not all cyclic genes identified to date belong to the Notch signalling pathway. However, a detailed microarray analysis in the mouse PSM established that these cyclic genes belong to only three signalling pathways, namely Notch Wnt and FGF signalling pathways (Figure F) (Dequéant et al., 2006). Moreover, a recent study suggested that genes from these two other pathways may also show dynamic expression in the caudal PSM/tailbud of the chick and zebrafish (Krol et al., 2011).

6. The Wnt pathway

Wnt ligands are a family of secreted glycoproteins which have a high number of conserved cysteine (cys)-residues. In vertebrate there are 19 Wnt ligands, and several of these have been described as essential for the process of somitogenesis (See below). The first Wnt gene to be characterised was *Wnt1*, identified in 1982 by Nusse and Varmus in mouse. Wnt proteins are secreted and bind to Frizzled proteins (Fz), which are seven-pass transmembrane receptors (in mammals there are 10 Frizzled receptors) with an extracellular N-term cys-rich domain (CRD) (Bhanot et al., 1996). This interaction appears to be promiscuous, i.e. one Wnt ligand can bind to many Fz proteins, and vice-versa.

In the absence of Wnt binding, the stability of β-catenin, the effector of the canonical Wnt cascade, is regulated by the destruction complex. The tumour suppressor protein Axin is the central component in the destruction complex, as it interacts with other complex components: APC, components of CK1 (α, δ, and ε) and Glycogen synthase kinase 3 (GSK3) (α and β) kinase families and β-catenin. CK1 and GSK3 α and β sequentially phosphorylate β-catenin at conserved serine and threonine residues near the N-terminus. β-catenin is then recognised and ubiquitinated by β-TrCP (β-transducin repeat-containing protein), a component of an E3 ubiquitin ligase complex, thus targeting β-catenin for destruction by the proteasome (Aberle et al., 1997).

Once bound to Wnt, Fz binds to a single-pass transmembrane molecule called lipoprotein receptor-related protein 5/6 (LRP5/6) in vertebrates, forming a trimeric complex (Pinson, Brennan, Monkley, Avery, & Skarnes, 2000). Dickkopf (Dkk) proteins act to inhibit Wnt signalling by binding to LRP5/6 to form crosslinks to other transmembrane molecules (B.

Mao et al., 2002), and thereby inactivating LRP5/6. Soluble Frizzled proteins (sFRPs) are also Wnt inhibitors and act by sequestering Wnt ligand proteins, as they resemble the ligand-binding cys-rich domain of Fz proteins (Moos, 1996). Once the ligand is bound, the Fz/LRP complex activates canonical Wnt signalling: Fz interacts with Dishevelled (Dvl), a cytoplasmic protein which acts upstream of other Wnt components GSK3 and β-catenin; Dvl is then phosphorylated by casein kinase 1 (CK1) (Takada et al., 2005). Wnt binding also induces phosphorylation of the cytoplasmic domain of LRP, which regulates the docking of Axin2 protein. Phosphorylation of LRP occurs by GSK3 and CK1γ (Casein kinase 1 γ), thus regulating the docking of Axin2. Since Axin2 is recruited away from the destruction complex, β-catenin is released from the destruction complex, and thus accumulates in the cell cytoplasm. β-catenin is then translocated to the nucleus (the mechanism by which this occurs is currently unknown). β-catenin interacts with transcription factors LEF1/TCF1 (lymphoid enhancer factor/ T-cell specific transcription factor) at the N-terminus domain (Behrens et al., 1996; Molenaar et al., 1996; van de Wetering et al., 2002), where it displaces the transcription factor Groucho from LEF1/TCF1 to promote Wnt target gene transcription (Daniels & Weis, 2005). In the absence of Wnt binding, TCF1 forms a complex with Groucho to act as a transcriptional repressor of Wnt target genes (Figure D).

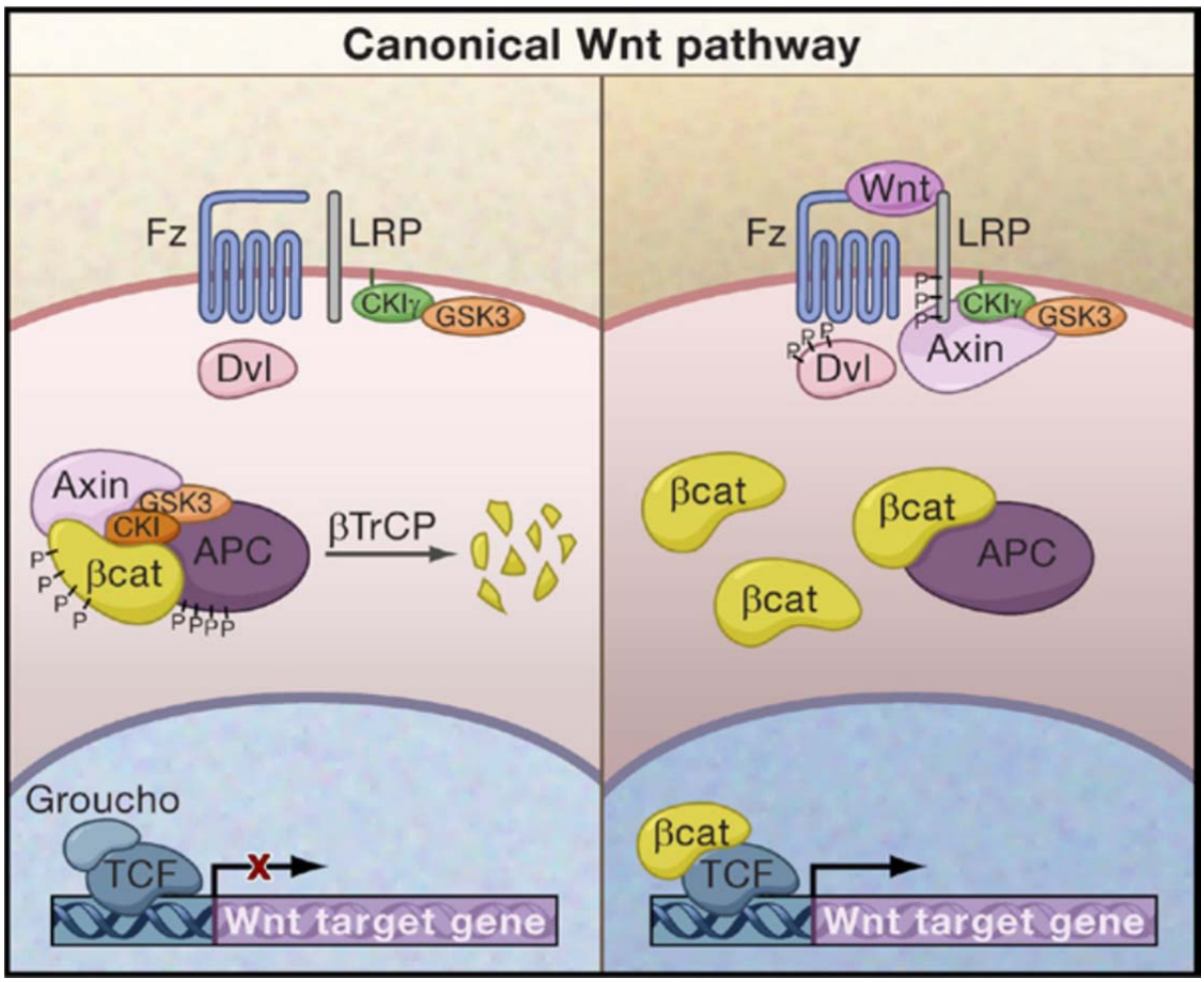


Figure D: The canonical Wnt signalling pathway

When the Wnt receptor complexes are unbound to Wnt ligands, β-catenin is phosphorylated by CK1 and GSK3 in the destruction complex, and is subsequently ubiquitinated and targeted for degradation. When a Wnt ligand binds to the Fz/LRP co-receptor complex, canonical Wnt signalling is activated: Dvl phosphorylates components of the destruction complex, causing it to dissociate, which then allows β-catenin to translocate to the nucleus, where it interacts displaces Groucho and induces TCF1 to activate transcription of Wnt target genes (taken from Clevers, 2006).

6.1 Wnt in somitogenesis

Mice carrying a null mutation in the Wnt ligands Wnt3 or Wnt3a reveal the essential role that Wnt plays in gastrulation and mesoderm formation. In mice embryos which harbour the Wnt3a hypomorphic allele *vestigial tail* (vt), in which *Wnt3a* expression is lost after stage E9.5, embryos studied at E10.25 revealed a down regulation of *Axin2*, *fgf8* and *Brachyury* in the posterior PSM. It was also shown that the expression domain of *mUncx4.1*, a marker of the caudal halves of segmented somites, was broadened in the anterior tail region, while the posterior tail region showed a 'salt and pepper'-like pattern of expression indicating that segmentation was impaired, as there were no morphological signs of somitogenesis (Aulehla et al., 2003).

Studies have also confirmed the importance of the Wnt ligand *Wnt5a* for somitogenesis and the extension of the A-P body axis in mutant mice embryos. Because *Wnt5a* itself is not expressed in the forming somites, it has been hypothesised that the effects from a loss of function mutation of *Wnt5a* of a shorter body axis and smaller somites is derived from the requirement of *Wnt5a* in the PSM and primitive streak. The reduced number of proliferating cells in the primitive streak mesoderm indicates the importance of *Wnt5a* for the proliferation of progenitor cells during gastrulation (Yamaguchi et al., 1999).

It has also been shown that transcription factors downstream of Wnt signalling are essential for mesoderm formation (and therefore somitogenesis): the T-box homeodomain transcription factor Brachyury (T) is thought to be required for generation of mesoderm, as loss of the T gene results in a failure of axis development and subsequently arrested somite formation (Herrmann, 1991; Wilkinson et al., 1990).

More specifically in the paraxial mesoderm from which somites arise, over the last decade it has been shown that while the Wnt ligands themselves are expressed in a caudorostral gradient, the mRNA of Wnt target genes such as *mAxin2* (Aulehla et al., 2003), *mNkd1* (Ishikawa et al., 2004), and *mDact1* (Suriben et al., 2006) oscillate across the mouse PSM. Interestingly, these genes encode transcriptional repressors, indicating that negative feedback loops of Wnt targets similar to those of Notch signalling operate in the PSM. Moreover it has been reported that components of this pathway also oscillate in the PSM of mice and fish (Krol et al., 2011). It has been shown that Wnt target genes oscillate out of phase with Notch targets (Dequéant et al., 2006), and that the inhibition of Wnt signalling slows the oscillation of target genes in the mouse and chick PSM, indicating that Wnt signalling is a key regulator of clock pace (Gibb et al., 2009). This fits with the observation that oscillations are slower in the rostral PSM, where nuclear β-catenin concentrations are reduced (Aulehla et al., 2008). Moreover, the pace of somitogenesis and clock oscillations slow in late stage embryos concurrent with a drop in levels of Wnt ligand in the PSM in both mouse and chick (Tenin et al. 2010) How Wnt regulates clock pace is currently being investigated. In contrast, up regulating Wnt activity does not change the pace of the segmentation clock (Aulehla et al., 2008; Dunty et al., 2008; Gibb et al., 2009), indicating that oscillations are already proceeding at a maximum pace, and/or that Wnt activity is already saturated in the PSM.

Another vital role that Wnt signalling plays in somitogenesis is to maintain cells in the caudal PSM in an immature non-determined state. This role is mediated through the FGF signalling pathway. A more detailed explanation of this function is given below in Section 10, where I discuss the wavefront of maturation.

7. The FGF Pathway

The FGF pathway is crucial during embryogenesis since it drives mesoderm and neuroectoderm specification, control of morphogenetic movements, anterior-posterior patterning, and somitogenesis. There are 22 FGF extracellular ligands in vertebrates (Ornitz, 2000), which signal through tyrosine kinase receptors called FGF receptors (FGFRs). There are four *fgfr* genes (1-4) in vertebrates each of which undergo alternative splicing, generating even more complexity of receptor subtype and each of these have different affinities to ligands (X. Zhang et al., 2006). FGF ligands bind to the extracellular domain (ECD) of FGFR with heparin sulphate to form a 2:2:2 FGF:FGFR:heparin dimer. This dimerization causes trans-phosphorylation of specific tyrosine residues in the cytoplasmic domain of the receptor, causing the activation of Ras/ERK (extracellular-signal-regulated kinase) pathway and the PI3K (Phosphatidylinositol 3-kinase) /Akt pathway cascades.

The Ras/ERK pathway is the most common pathway downstream of FGF signalling, and involves the lipid-anchored docking protein FRS2 (Fibroblast growth factor receptor substrate 2) to bind to FGFR1. After FGF binding, multi-phosphorylated FRS2 acts as a site of multi-protein complex assembly that activates the Ras-ERK and PI3K/Akt pathways. Grb2(Growth Factor Receptor-Bound 2), an adapter protein, and protein tyrosine phosphatase (PTP) Shp2 (Src Homology Phosphatase 2) bind to FRS2 tyrosine phosphorylation sites. Grb2 forms a complex with the guanine nucleotide exchange factor: Son-of-sevenless (SOS) via its SH3 (*Src (*Sarcoma) Homology 3) domain. SOS then activates Ras by GTP (Guanosine 5'-Triphosphate) exchange due to its proximity to the membrane-bound Ras. Once Ras is in the active GTP-bound state, Ras interacts with as well as several other effector proteins, activating the ERK signalling cascade, which then leads to the

phosphorylation of transcription factors such as c-myc (cellular-myc) and AP1 (activator protein-1) (Figure E).

7.1 FGF in somitogenesis

It was recently shown that the PSM-specific loss of FGF4 and FGF8 ligands the mouse embryo leads to a loss of cyclic gene expression and premature differentiation of the PSM (Naiche et al., 2011). Upon restoring Wnt activity, although some PSM progenitor markers are partially restored, the tissue still differentiates prematurely, indicating that FGF acts to maintain non differentiated cells in the caudal PSM in an immature state, independently of Wnt signalling. In addition to these potential roles of initiating oscillations and maintaining cells in an immature state in the caudal PSM, FGF target genes were found to oscillate in the mouse PSM: *mSpry2*, *mDusp6* (Dequéant et al., 2006), *mSpry4* (Hayashi et al., 2009), and *mDusp4* (Niwa et al., 2007); all of which oscillate in synchrony with Notch targets. It was recently reported that FGF genes also oscillate in the chick and Fish PSM (Krol et al., 2011).

The FGF pathway also plays another vital role in the PSM of chick, mice and fish embryos. This role is to regulate the size of somites and the position of boundary formation (see Section 10 for more detail).

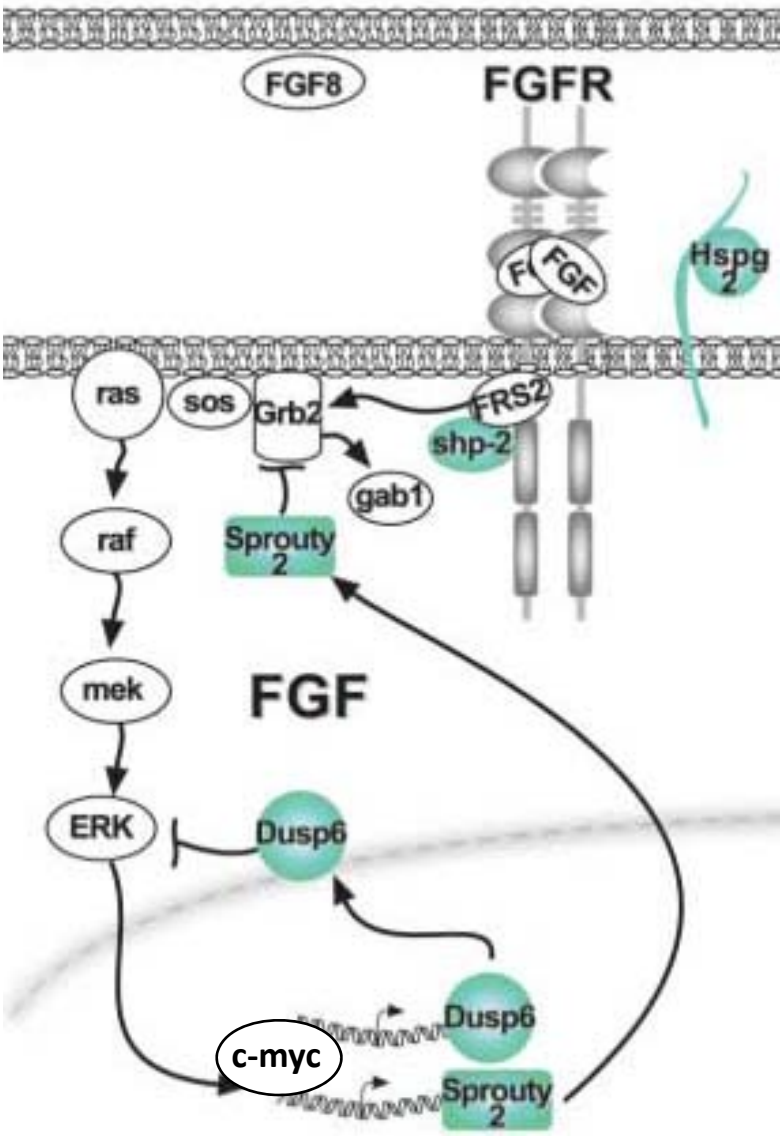


Figure E: The FGF/ERK Signalling Pathway

FGF signalling occurs when the FGF ligand binds to the receptor at the plasma membrane. This binding initiates phosphorylation of FGFR and FRS2, causing Grb2 to bind to the pospho-tyrosine of FRS2. The subsequent formation of a Grb2/SOS complex then activates Ras and causes a downstream cascade of Raf/MEK/ERK, and subsequently allows the downstream transcription of transcription of FGF target genes through the phosphorylation of transcription factors such as c-myc (figure adapted from Dequéant et al., 2006).

8. Interplay between Notch, Wnt and FGF in Somitogenesis

One of the critical question which remains in this developmental process is: how do pathways communicate and co-regulate each other in order to allow the segmentation clock to function? Most studies of cross-talk in the PSM have been performed in mutant mouse studies and drug inhibition experiments (Pourquié, 2011).

There is evidence from multiple reports to suggest that Notch and Wnt signalling co-regulate each other in the PSM. For example, inhibiting Notch signalling in mice leads to the strong downregulation of the Wnt target *Axin2* (Ferjentsik et al., 2009). Furthermore, in Dll1-/- mice, the levels of *Axin2* are affected (Aulehla et al., 2003); and the Wnt target *mNkd1* does not oscillate in Hes7-/- embryos (Ishikawa et al., 2004). This data suggests that Notch signalling regulates Wnt signalling in the PSM, however, there is also evidence that Wnt reciprocally regulates Notch signalling in the PSM. When *Axin2* is misexpressed, it causes *Lfng* to be up regulated, thus disrupting segmentation (Aulehla et al., 2003); Notch1 expression is lost in LEF1-/-;TCF-/- mice (Galceran et al., 1999); and in vestigial tail (vt) mutant mice, which lacks Wnt3a in the PSM, the cyclic expression of Notch regulated clock genes *mLfng* and *mNrarp* is disrupted (Aulehla et al., 2003; Sewell et al., 2009).

The mechanism by which this co-regulation occurs is currently poorly understood in the PSM. There are some very interesting points of potential cross-talk between the pathways, however. One example is Notch-regulated ankyrin repeat protein (Nrarp), which has been implicated in the regulation of Notch and Wnt signalling by destabilising NICD and stabilising LEF1 in the context of *in vitro* tissue culture experiments (Ishitani et al.,,2005; Lamar et al., 2001). In the mouse chick and fish *Nrarp* expression oscillates across the PSM although this

dynamic expression is Notch dependent in chick, mouse and fish but Wnt dependent only in the mouse. However, since the loss of Nrarp does not affect either somitogenesis or cyclic gene oscillations in any of the three species this factor clearly does not provide the key focal point of crosstalk between these pathways (Wright et al., 2009).

Another point of cross-talk is the regulation of *mDll1* transcription by Wnt signalling. It has been shown that Wnt regulates *mDll1* expression through LEF/TCF factors that were shown to cooperate with TBX6 (T-Box 6), in order to activate *mDll1* transcription by binding to the promoter in *in vitro* luciferase assays. These findings were verified through the mutation of either the T-box or LEF/TCF binding sites which abolished reporter gene expression both *in vivo* and *in vitro* (Galceran et al., 2004; Hofmann et al., 2004). It was also shown that induced expression of Lef-β-catenin in fibroblast cells causes endogenous Dll1 to be expressed (Galceran et al., 2004).

There is also clear cross talk between Notch and FGF signalling pathways: the main example of this is that HES7 regulates the transcriptional oscillation of the FGF target *Dusp4* in the mouse PSM (Niwa et al., 2007), which is consistent with the observation that Notch and FGF clock gene targets oscillate in phase across the mouse PSM. Moreover, FGF is required to initiate *Hes7* transcription in the mouse tail bud (Niwa et al., 2007). This indicates that Notch and FGF activity are intricately linked in order to initiate oscillations in the PSM at least in mouse. *Lfng* expression is absent in RBPj-/- mice, which suggests that Notch signalling is required for *Lfng* expression (Ferjentsik et al., 2009), although *Lfng* expression is maintained in *Fgfr1*-/- mice, this expression is non-cyclic, showing that FGF signalling is required for *Lfng* oscillations (Wahl et al., 2007). This contrasts with findings which show that short term pharmacological inhibition of FGF signalling does not disrupt the oscillations

of *Hes7* or *Lfng* (Gibb et al., 2009). There has also been some recent evidence to suggest that the FGF pathway is required for Wnt signalling in the zebrafish embryo: by using FGF inhibitors, it was suggested that FGF signalling may act to activate Wnt signalling by inhibiting the expression of Wnt antagonists such as *dkk1* (Stulberg et al., 2012).

Further work is therefore required to account for these observations and also to investigate the degree of crosstalk between all three pathways in species other than mouse.

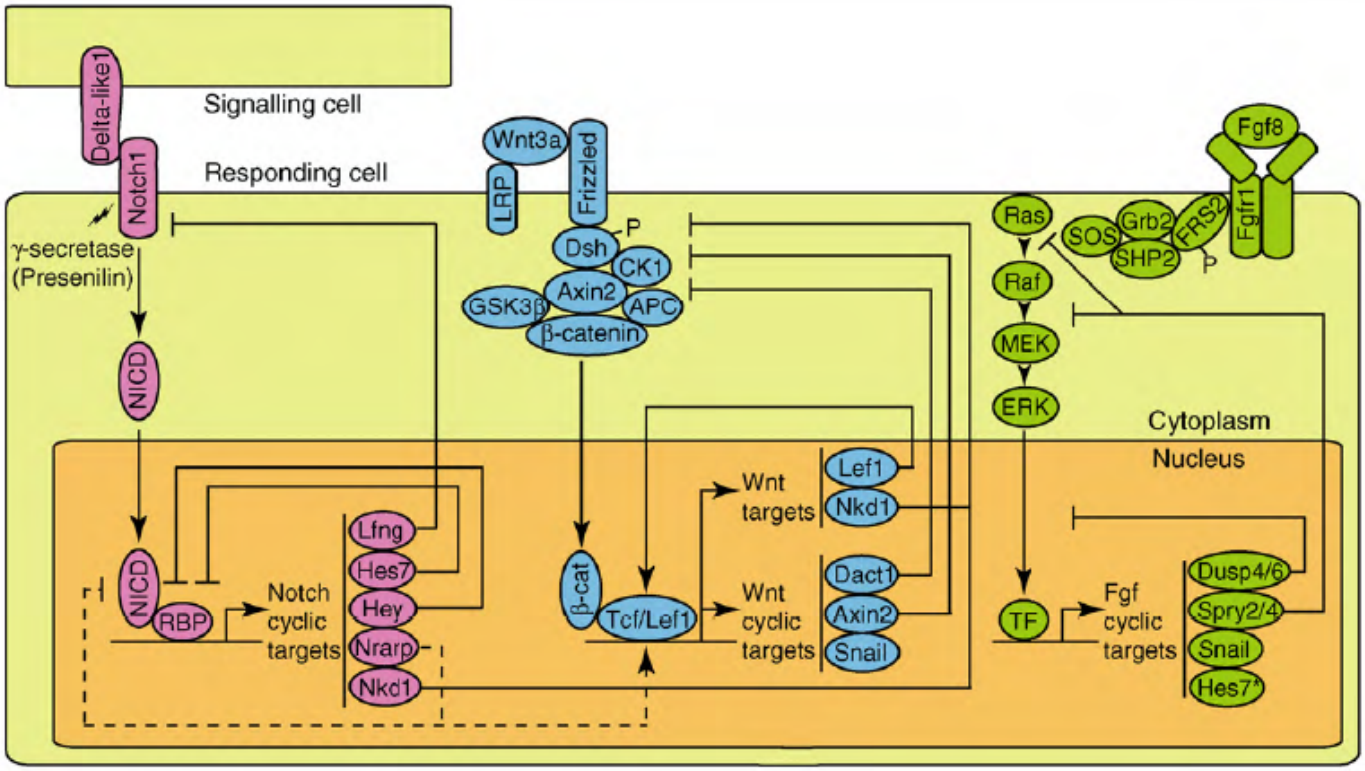


Figure F: The mouse segmentation clock

Notch, Wnt and FGF pathways all have cyclic genes in the PSM. Cyclic genes of the Notch and FGF pathways oscillate in synchrony, and asynchronously with Wnt cyclic targets. Several of these genes are involved in negative feedback loops (Hes7 is FGF-dependent in the tail bud only). Dashed lines indicate interactions that have only been shown in tissues outside the PSM. *Hes7 is regulated by the FGF pathway in the tail bud only (figure taken from Gibb et al., 2010).

9. The Zebrafish Oscillator

In the zebrafish model, it has traditionally been thought that the cyclic genes all belonged to the Notch pathway, such as *her1, her7, her11, her12, her15* (homologous to *cHairy1*), and *deltaC* , although recent microarray data has postulated that Wnt and FGF targets may also by dynamic in the caudal PSM/tail bud of the fish (Krol et al., 2011). Mutants for several Notch pathway genes identified in a genetic screen (van Eeden et al., 1996) show disrupted somitogenesis: Notch1A, DeltaC, and DeltaD, and Mindbomb (which encodes a ubiquitin ligase, required for Delta endocytosis and Notch activation). In these Notch zebrafish mutants, oscillations of Notch cyclic target genes in the PSM progressively become desynchronised and their expression appears as a 'salt and pepper' pattern in the PSM (Jiang et al., 2000).

Gain-of-function and loss-of-function studies with Her proteins have led to the formation of a model wherein Her1 and Her7 generate negative feedback loops by repressing their own transcription (Lewis, 2003). In this model, there is a defined time delay from initial transcription of *her* genes to Her proteins binding to their own promoter, which establishes the oscillations (Figure G). Mathematical modelling reveals that genes and proteins must be unstable for the model to work: embryological studies have shown that the established parameters of gene and protein stability and transcriptional/translational delay were accurate. However, embryos still form abnormal somites in the absence of HER1 and HER7, so redundancy with other her genes may account for this (Henry et al., 2002). HER1/7 represses the transcription of *DeltaC*, and trigger dynamic patterns of Notch activity in the PSM (Giudicelli et al., 2007). MO-knockdown of *her1/7* in zebrafish embryos results in

accelerated oscillations of neighbouring wild-type cells, causing an anterior shift in somite boundaries (Henry et al., 2002).

It was also shown that loss of *deltaC* and *her7* by morpholino (MO)-knockdown caused a failure of cyclic domains to initiate, resulting in defective segmentation, a similar phenotype to the loss of *her1/7* (Oates et al., 2005).

This raised the question of whether Notch signalling is also required for her oscillations. Notch inhibition by DAPT treatment causes eventual somite defects, although initial oscillations of *her* genes (equivalent to gastrulation stages in chick) are unaffected (Riedel-Kruse et al., 2007); DAPT pulse experiments showed that once DAPT was removed, somites were able to form again (Ozbudak & Pourquié, 2008; Riedel-Kruse et al., 2007). Importantly, the time taken for this recovery after removing DAPT was similar to the time taken for somitic defects to occur following exposure to DAPT, indicating that the primary role of Notch signalling in the PSM is to maintain the synchrony of oscillations (Riedel-Kruse et al., 2007).

It has been established that *her* expression is not abolished when the Notch-inhibiting drug DAPT is added, and initial oscillations do not require Notch, However, when Notch1A is up regulated in the PSM, this causes an up regulation of *her1* in the PSM, which indicates that *her1* may be a target of Notch signalling, and is consistent with observations made in other model systems (Takke & Campos-Ortega, 1999). Moreover, further similarities with observations in mouse come from the observation that *her13.2*, a *Hes*-related gene downstream of FGF, is required for *her*1/7 oscillations recapitulating the cross talk between notch and FGF pathways reported in the mouse PSM. HER13.2 forms a heterodimer with

her1, and therefore enhances its ability to bind to the her1 promoter site (Kawamura, et al., 2005).

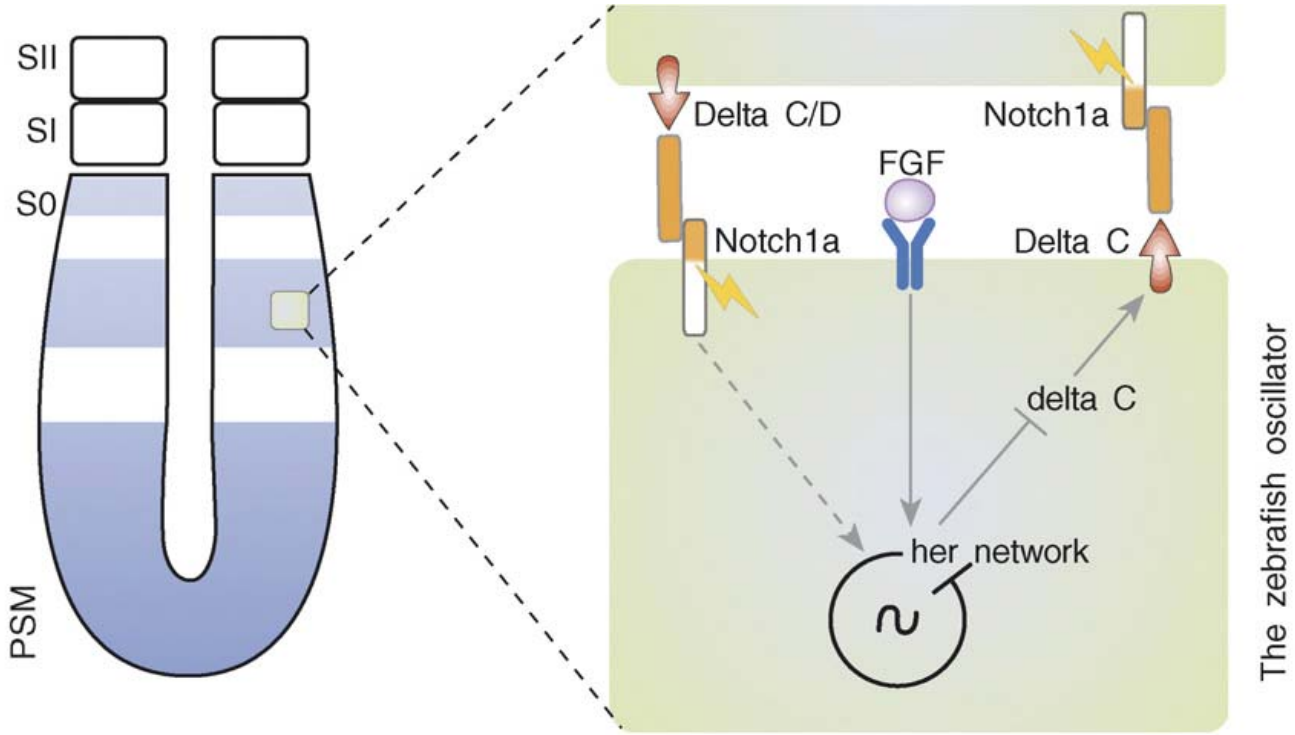


Figure G: The Zebrafish Segmentation Clock

In the zebrafish vertebrate segmentation clock, the Notch signalling pathway couples the cell-autonomous oscillations of the *her* network between neighbouring cells. Notch and FGF pathways regulate the expression of the *her* genes, which in turn regulates the expression of deltaC, which also oscillates and subsequently activates Notch signalling in neighbouring cells (taken from Ozbudak & Pourquié, 2008).

10. Wavefront of Maturation and the Determination Front

According to the clock and wave front model the oscillator interacts with a wavefront of maturation in the PSM. How does this "wavefront" manifest molecularly in the PSM? There is now a wealth of data in the literature to support a highly conserved molecular gene regulatory network for this "wavefront".

In the PSM there are two opposing molecular gradients: an anterior-to-posterior gradient of retinoic acid (RA), and a posterior-to-anterior gradient of FGF8 and Wnt3a (Figure H). The point at which these two gradients intersect is known as the "determination front". RA cannot be visualised directly, but its activity can be detected in a reporter mouse line for RARE-LacZ (Rossant et al., 1991). The highest expression levels for RA are in the rostral PSM and somites, with its expression absent in more caudal regions of the embryo. This correlates with the expression of RA metabolism enzymes, e.g. Raldh2 (Niederreither et al., 1997). In contrast, Cyp26A1, the cytochrome P450 enzyme involved in RA degradation, is expressed in the tail bud (Fujii et al., 1997), therefore the RA gradient is spread over a long distance, kept in check by synthesising and degrading enzymes, creating a 'source/sink' model.

In contrast to the source/sink model, the FGF8 protein gradient, which is highest in the tail bud and in the PSM is due to a mRNA decay mechanism (Dubrulle & Pourquié, 2004). *De novo* transcription of *fgf8* occurs only in the tail bud, so that the further anterior the cells are the larger the degree of mRNA degradation within those cells. This mechanism establishes a concentration gradient in the PSM (Dubrulle & Pourquié, 2004). In order for the mechanism to work, cells have to leave their place of origin where the ligand is

produced, and then the ligand concentration can decrease over time. This is known as the "cell lineage transport" model (Ibañes et al., 2006). FGF8 binds to FGF Receptor 1 (FGFR1) (the only FGF receptor in the mouse PSM (Wahl et al., 2007)), thereby triggering FGF signalling via downstream pathways Ras/ERK and Akt/PI3K (Böttcher & Niehrs, 2005).

In the mouse embryo, the signalling gradients of FGF8 and Akt (kinase downstream of PI3K) correlate in the PSM (Dubrulle & Pourquié, 2004). In the chick and zebrafish embryos, gradients of FGF8 and ERK correlate (Delfini et al., 2005; Sawada et al., 2001). The gradient of Akt/ERK activity is responsible for the boundaries of expression for FGF targets such as Dusp4 (Niwa et al., 2007), Dusp6 (Dequéant et al., 2006), and Snail1 (Dale et al., 2006) or Pea (Roehl & Nüsslein-Volhard, 2001) in the PSM. The wavefront of maturation predicted in the Clock and Wavefront model is believed to rely on the fact that cells become progressively anteriorly displaced in the PSM during the process of somitogeneiss and concomitant with this they are exposed to changing levels of these opposing signalling gradients that influence their maturation status as described below. As cells enter the posterior PSM, levels of the proliferative factors Wnt and FGF are high, and consequently the cells are maintained in an undifferentiated, non-determined state. However, as cells become more rostrally displaced in the PSM (with the continuous recruitment of more cells into the caudal PSM from the tail bud and budding off of new somites from the rostral PSM) the cells become exposed to progressively lower levels of FGF8/Wnt3 (and begin to be exposed to RA signalling) until the point that they reach the anterior third of the PSM whereupon cells hit the so-called determination front and initiate their segmentation program (Dubrulle et al., 2001). This level of non-commitment in the caudal PSM versus commitment to a somitic compartment in the anterior PSM was elegantly demonstrated by

a series of intricate tissue inversion experiments in these two regions performed by Dubrulle et al. In 2001, Dubrulle et al. inverted chunks of PSM which were one somite in length in these two domains (rostral and caudal PSM), and found that this inversion only disrupted somitogenesis anterior to position S-IV. Caudal to this point the tissue adapted to the inversion thereby indicating that caudal to S-IV cells are non-determined as to segmental identity and boundary position. They went on the demonstrate that FGF8 was the molecular player regulating this commitment by grafting FGF8-coated beads in the chick PSM tissue and, they found that segmentation of only the cells posterior to S-IV were affected. This indicated that anterior to the bead cells were committed to their segmental identity and size and no longer receptive to FGF influence (Dubrulle et al., 2001). This was also observed to be the case for the zebrafish, where following the treatment of zebrafish embryos with an FGF8 inhibitor, the first 4-5 pairs of somites were normal, and only after these somites were any defects observed (Sawada et al., 2001). The point at which specification must occur is the point at which the concentration of FGF8 diminishes, and for reproducible somite size, the gradient must be sufficiently steep. The size of each somite is determined by the number of PSM cells which pass the determination front during the time taken for one cycle of the segmentation clock to be completed. The posterior PSM is made up of loose mesenchyme, but as the cells become more anteriorly displaced they also become progressively epithelised (Delfini et al., 2005).

The mesenchymal-to-epithelial transition (MET) in the PSM is thought to be repressed by the presence of the *snail* genes, which are expressed from the tail bud until the determination front in the manner of a clock gene (Dale et al., 2006), and down regulate *E-cadherin* transcription, thus preserving the mesenchymal character of the caudal PSM

(Carver et al., 2001). However, in the rostral PSM where FGF and Wnt signalling are down regulated, expression of oscillating *snail* expression ends, which correlates with the up regulation of adhesion molecules such as E-cadherin (Duband et al., 1987; Horikawa et al., 1999; Linask et al., 1998), as well as deposition of a basal lamina (Rifes et al., 2007), thus leading to the progressive epithelisation of the anterior PSM. Ultimately the newly formed somite is an epithelial ball of cells that buds off the anterior end of the PSM.

The difference between the anterior and posterior PSM, i.e. the intersection of which is the determination front, is characterised by the expression profile of specific genes: in the anterior PSM, the bHLH transcription factors MESP-1 and -2 are expressed (Saga, 2007; Takahashi et al., 2007) as well as *Paraxis*, a bHLH gene which is required for the epithelisation of somites (Burgess et al., 1996); whereas in the posterior PSM, *Mesogenin1* is expressed, with an anterior border of around S-II, which meets the posterior limit of *Paraxis* (Yoon et al., 2000). In mice which lack FGF4 and FGF8 in the PSM, the *Mesp2* expression domain is increased, indicating that FGF signalling normally represses *Mesp2* expression, thereby limiting the influence of this bHLH factor to bestow anterior segmental character to cells that are in the anterior third of the PSM (Naiche et al., 2011).

fgf8-/- mice do not display a somitic phenotype, which indicates that there could be a redundancy with the Wnt gradient and/or other FGF ligands (Reifers et al., 1998).

The positioning of the determination front is not solely reliant on the cell lineage transport model of mRNA degradation. It is also positioned by virtue of the fact that FGF8 and RA signalling act in opposition in the PSM (Diez del Corral et al., 2003). During early somitogenesis, the chick embryo has high levels of RA, which becomes down regulated in the posterior PSM (Maden et al., 1998). After treating the chick PSM with RA, *fgf8*

expression is down regulated (Diez del Corral et al., 2003), and reciprocally ectopic expression of FGF8 in the chick PSM causes a down regulation of Raldh2 (Delfini et al., 2005; Diez del Corral et al., 2003).

It is noteworthy that there are some controversial reports in the literature with respect to the requirement for RA in regulating the wavefront of maturation and the position of the determination front: One study reported that RA appears to be required for somitogenesis only in early stages, (Sirbu & Duester, 2006). Moreover this study suggested that RA appears to restrict FGF8 in the ectoderm, rather than the PSM itself (Sirbu & Duester, 2006). However, this is directly contradictory to the findings of Diez del Corral et al., who state that RA acts to inhibit FGF activity within the PSM, as well as in the neural tube (Diez del Corral et al., 2003). Because it is currently not possible to directly visualise RA, this discrepancy remains to be resolved. Furthermore, a report was published stating that the use of mouse reporters suggests that RA does not exist as a gradient, but rather stops abruptly in the anterior PSM (Vermot et al., 2005).

It is thought that the distance travelled by the wavefront during the time taken for one oscillation of the segmentation clock to complete, determines the size of prospective somites. The best way to test this theory would be to alter the progression of cells in the PSM without affecting the segmentation clock; however this remains impossible for the time being. Many studies have focused on altering the wavefront, such as disrupting RA signalling (Maden et al., 2000; Niederreither et al., 1999; Sirbu & Duester, 2006; Vermot et al., 2005; Vermot & Pourquié, 2005; Diez del Corral et al., 2003), incubating with exogenous RA (Moreno & Kintner, 2004), disrupting the Wnt signalling gradient (Nagano et al., 2006) and grafting Wnt-secreting cells(Aulehla et al., 2003). These studies have all shown that

when FGF8/Wnt is up regulated, the determination front is pushed anteriorly, resulting in the production of smaller somites (Aulehla et al., 2003; Delfini et al., 2005; Dubrulle et al., 2001; Sawada et al., 2001), whereas the up regulation of RA causes the opposite to happen: the Determination front is moved to a more posterior location, resulting in the formation of larger somites (Moreno & Kintner, 2004). This also occurs when FGF signalling is blocked in the PSM by pharmacological inhibitors (Dubrulle et al., 2001).

There are still many unanswered questions in this field, such as how do cells in the caudal PSM, which are located anterior to a grafted FGF8-coated bead, respond more strongly than cells which are situated caudal to the bead, at the same distance (Delfini et al., 2005). This muddles the interpretation of the bead experiments conducted by Dubrulle et al., and it could be linked with the observed phenomenon of asymmetric response to FGF8-soaked beads in the PSM: where cells anterior to the bead form smaller somites than cells posterior to the bead (Dubrulle et al., 2001). Other unexplained phenomena include how the interaction of FGF and Wnt signalling occurs to regulate the wavefront, how changes in FGF8 signalling affects the components of the segmentation clock, and how the clock and wavefront jointly control targets involved in somite specification and boundary formation at the determination front.

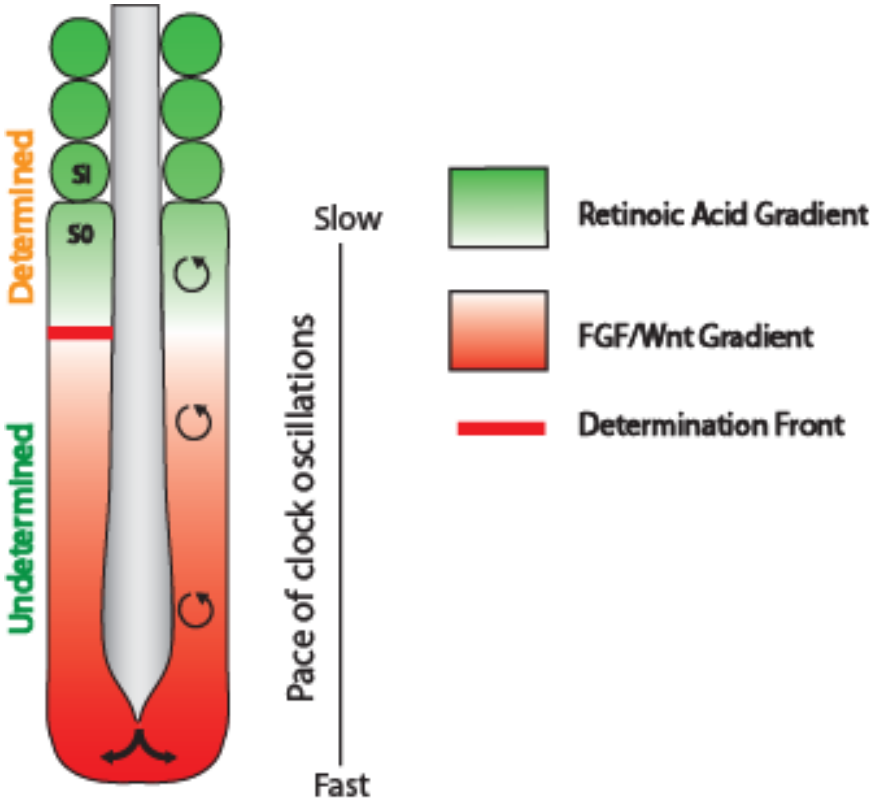


Figure H: The Determination Front

The Determination Front is marked by the intersection of the caudo-rostral gradients of FGF8 and β-catenin with the rostro-caudal gradient of RA activity. The region of the PSM anterior to the determination front contains cells which are segmentally determined, in which the clock oscillations are slowed down (adapted from Maroto et al., 2012)

11. Segmental Boundary Formation

Once the wave of transcription of clock genes reaches the anterior PSM, it is arrested at the future segmentation point, where temporal information is translated into a spatial pattern, manifest in the formation of the somites (Y Saga & Takeda, 2001). A somite is divided into a rostral and caudal compartment, which are determined before the morphological boundary is formed (Aoyama & Asamoto, 1988). The repression or activation of Notch is crucial in this process, as demonstrated when gain-of-function and loss-of-function of Notch signalling results in defective rostro-caudal patterning of the somites (Bessho et al., 2001a; Evrard et al., 1998; Feller et al., 2008; Takahashi et al., 2000; Zhang & Gridley, 1998). Mesp2 is a crucial component of rostro-caudal patterning in a somite, and is initially expressed in the anterior PSM in a one somite domain, which is then reduced to a half somite length via down regulation in the prospective caudal half (Figure I). This specifies the anterior half of the somite and Mesp expression is then lost from the somite as it buds off the anterior tip of the PSM. (Morimoto et al., 2005; Saga et al., 1997; Takahashi et al., 2000). Quite a lot is known about the regulation of Mesp in this dynamic sequence in the mouse PSM: *Mesp2* expression is dependent on TBX6 and NICD activity (Yasuhiko et al., 2006), and is negatively regulated by FGF and is therefore expressed in the FGF-negative rostral tip of the TBX6 expression domain in a one somite width (TBX6 is expressed throughout the entire PSM (Chapman et al., 1996)). Within that domain Mesp2 leads to Tbx6 degradation in a ubiquitin-dependent manner, causing a new Tbx6 anterior border, which defines the Mesp2 expression domain in the next cycle (Oginuma et al., 2008). Notch and Mesp2 also play roles in rostro-caudal patterning of somites: Dll1 and Psen1 mutant mice show rostralised somites (Hrabě de Angelis et al., 1997; Koizumi et al., 2001). Mesp2-/- mice show somites with no

rostral identity and which are entirely caudalised, showing that Mesp2 is required for anterior-posterior compartmentalisation of the somites (Saga et al., 1997; Takahashi et al., 2000). The question remains: how does Mesp2 activity become restricted to the rostral S-I compartment, and how does Notch activity become restricted to the caudal S0 compartment? MESP2 inhibits Notch signalling in part through the activation of LFNG in the rostral somite compartment: in this region of the PSM, MESP2 is responsible for the transcription of *Lfng*, whereas in the posterior PSM the Notch signalling pathway is responsible for *Lfng* expression (Morimoto et al., 2005). It was thought that LFNG may be solely responsible for the down regulation of Notch activity in the rostral compartment of the somite; but in a series of experiments using Mesp2-/- mice with *Lfng* expressed under the Mesp2 promoter Notch activity was still present, showing that MESP2-induced expression of *Lfng* is involved but is not sufficient to down regulate Notch activity in this area (Oginuma et al., 2010).

Maml1 (Mastermind 1) is an essential component of Notch signalling, required for chromatin-dependent transactivation by the NICD/RBPj enhancer complex in vitro, which then recruits the proteins CBP and p300 to promote the nucleosome acetylation of Notch promoters (Fryer et al., 2002). Mesp2 suppresses Notch via the destabilisation of Maml1 at the post-transcriptional level, which prevents the formation of the NICD/Mam/RBPj complex, and therefore acts as a final 'off' switch for Notch signalling (Sasaki et al., 2011). In the absence of this activator complex forming, Notch target gene transcription is then inhibited by the repressive activity of RBPj. In the caudal somite, where Mesp2 levels are low, the activator complex is able to form and thereby Notch target genes are expressed and this bestows this region of the somite with caudal identity. Maml1 is a co-activator of β-

catenin and p53 at their target sites (Alves-Guerra et al., 2007; Zhao et al., 2007), and therefore Mesp2 could regulate other pathways such as Wnt through Mam1. In agreement with this, the transcription of Wnt target genes Lef1 and Axin2 is up regulated in the anterior PSM of Mesp2-/-mice, indicating that Wnt may be regulated by Mesp2 in the same manner as Notch (Aulehla et al., 2008).

The Ripply protein family are also required for rostro-caudal patterning in several species (Chan et al., 2006; Kawamura et al., 2005; Morimoto et al., 2007). Ripply1/2 proteins, which are downstream targets of Mesp2, recruit Groucho/TLE co-repressor to suppress Tbx6-mediated Mesp transcription, and in Ripply-/- mice the anterior boundary of Tbx6 is expanded, similarly to the Mesp2-/- embryo (Takahashi et al., 2010). This means, therefore, that a double negative feedback loop exists whereby Mesp2 is indirectly inhibited by Ripply1/2 proteins by repression of Tbx6 protein expression in the rostral PSM.

The latest additional facet postulated to this model is that the posterior NICD domain moves anteriorly through the PSM and narrows, whilst at the same time phospho-ERK (pERK) expands from the caudal tip of the PSM anteriorly, covering and inhibiting the NICD-dependent transcription of *Mesp* genes; after segmentation, pERK is down regulated in the rostral PSM, and NICD induces *Mesp* gene expression in S-I; this model postulates, therefore, that Notch oscillations are responsible for the segregation of a group of synchronised cells, while the FGF oscillations cause the release of synchronised cells for somitogenesis, thereby regulating the pace of the segmentation clock, and uniting the clock and wavefront processes (Niwa et al., 2011).

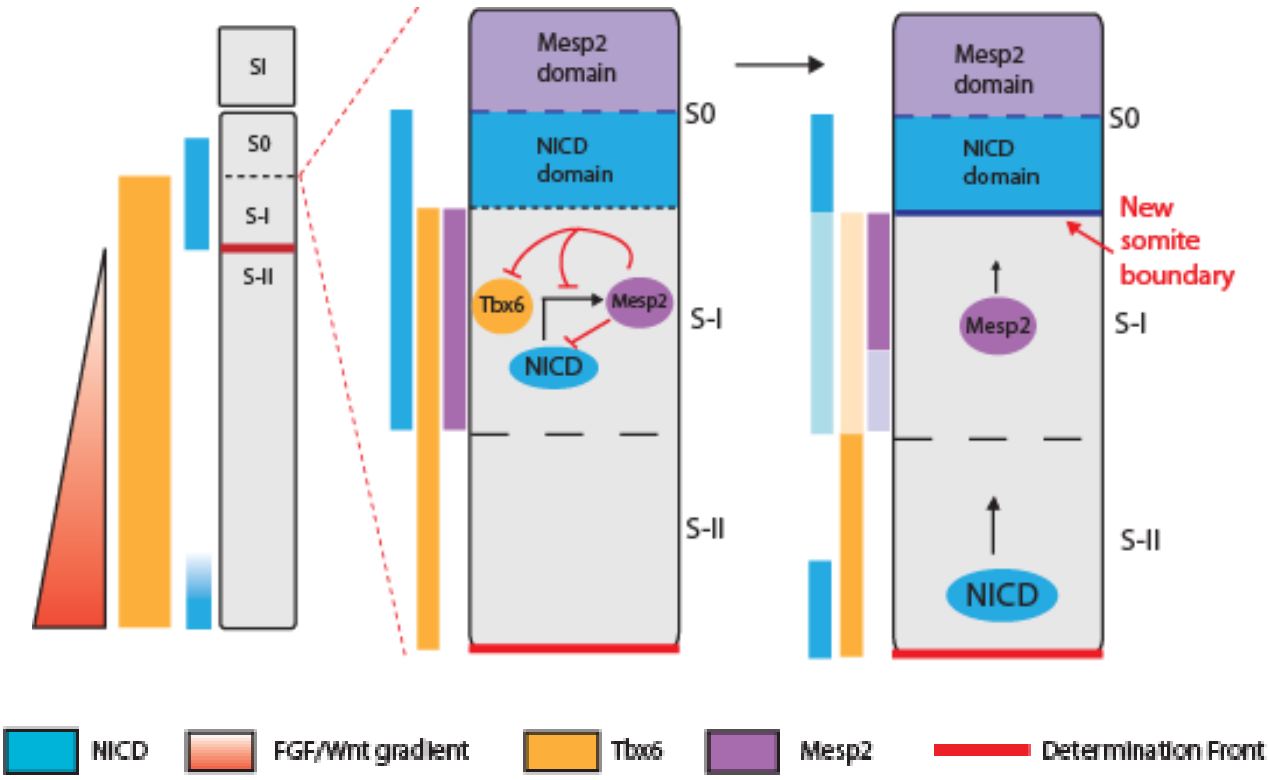


Figure I: Somite boundary formation

The Notch signalling pathway triggers a genetic cascade in the anterior PSM which generates the new somite. Mesp2 is activated by NICD in a Tbx6-dependent manner in S-I. FGF activity represses Mesp2 in cells which are posterior to the determination front. Mesp2 then causes the down regulation of Tbx6 and NICD, therefore limiting its expression domain to the anterior half of the prospective somites, whereas Notch activity is restricted to the posterior half (adapted from Maroto et al., 2012).

12. Somite Compartmentalisation

The division of the PSM into somites is not needed for differentiation of its derivatives, but it is essential for the proper organisation of these derivatives, especially the vertebral column (Burgess et al., 1996), and hypaxial portion of the myotome (Wilson-Rawls et al., 1999).

Somitogenesis involves the transformation of segments into epithelial somites that are separated by intersomitic furrows (Figure J). As described above, the anterior-posterior polarity of the somite is established by Notch signalling. Boundaries between cells of anterior and posterior identity in adjacent somites are established in morphological furrows between cells undergoing MET. In the rostral PSM, the Eph family of receptor tyrosine kinases and Ephrin ligands show Notch-dependent segmental expression (Barrantes et al., 1999; Durbin et al., 2000). Eph receptors are membrane-spanning proteins that are involved in many intercellular signalling processes that occur throughout development. Class A Ephrins have a GPI linkage to the cell membrane, and preferably bind to EphA receptors. Class B Ephrins are transmembrane ligands with an intracellular domain and preferentially bind to EphB receptors (Thorsteinsdóttir et al., 2011). EphA4 is the only receptor which binds to both classes of Ephrins (Gale et al., 1996). Eph/ephrin signalling is bidirectional (Cowan & Henkemeyer, 2002), resulting in repulsion between cell populations (Mellitzer et al., 1999), as evidenced by driving cell sorting and boundary formation in the zebrafish hindbrain (Cooke et al., 2001; Xu et al., 1999). Disruption of Eph/ephrin signalling leads to a lack of intersomitic furrow formation (Durbin et al., 1998). It was shown that the zebrafish ‘fused somites’ mutant (fss/tbx24), which has disrupted Eph/ephrin signalling, as well as causing a lack of boundaries; the restoration of EphA4/ephrin signalling in this mutant

results in the rescue of morphologically distinct boundaries. This led the authors to believe that Eph/ephrins have a role as intercellular signalling molecules that establish cell polarity during MET in the PSM cells (Barrios et al., 2003).

Cells at the next-forming boundary become specified to make a morphological gap on their anteriorly positioned cells within the PSM (Sato et al., 2002; Takahashi & Sato, 2008). Chimeric studies using *Mesp1/2* double-null cells in the mouse embryo have revealed that these cells fail to form a segmental border, as well as fail to contribute to either epithelial somite or dermomyotome formation. This indicates that MESP factors are essential for morphological boundary formation, and because in these chimeric mice there was no boundary formed by locally distributed wild type cells, which indicates that MESP acts non-cell autonomously to allow border formation (Takahashi et al., 2005). It has also been demonstrated through *in ovo* electroporation and transplantation techniques that LFNG restricts activity of Notch signalling to posterior of S-I in the PSM: following this, the Notch-activated cells signal to the cells anterior to them, inducing them to become segregated and eventually epithelialized (Sato et al., 2002).

Rho family member Cdc42 is critical for the MET of cells, which are juxtaposed to the forming gap (Nakaya et al., 2004). cMeso1 (chicken homologue of Mesp2) has been shown to up regulate EphA4 in cells which are posterior to the forming boundary. This then activates EphrinB2-reverse signals in anteriorly juxtaposed cells, which is sufficient to cause cell autonomous gap formation and cellular epithelialisation. During this process, Cdc42 needs to be repressed via tyrosine phosphorylation of Ephrin B2 (Watanabe et al., 2009). In border cells posterior to the next-forming boundary, cMeso1 up regulates Paired Box gene 2 (Pax2), PAPC (protocadherin), Sox9 (SRY (sex determining region Y)-box 9), and EphA4

(ephrin type-A receptor 4); and communicates with cells anterior to the boundary. The Ephrin reverse signal generated in anterior cells then causes gap formation and MET to occur concomitantly in a cell autonomous manner, but another as yet unidentified, factor generated by the Ephrin reverse signal, is required for boundary formation. The EphA4 forward signal is required for MET, but is dispensable for boundary formation (Watanabe et al., 2009).

Integrins are a class of cell adhesion receptors (Legate et al., 2009) which bind to extracellular matrix (ECM) proteins such as collagens and fibronectins (FNs). Loss-of-function experiments have established that integrins and FN are required for somite formation (Jülich et al., 2005; Kragtorp & Miller, 2007; Rifes et al., 2007; Sakai et al., 2001). Integrins are heterodimeric transmembrane proteins which link the ECM with the actin cytoskeleton, and consist of a α and β subunits (Bökel & Brown, 2002; Ginsberg et al., 2005; Hynes, 2002). Integrins are important for cell migration, survival and adhesion, and by acting as an allosteric switch, they cause bidirectional signal transduction across the plasma membrane. The 'outside-in' direction, which involves ECM signalling to the cytoplasm, causes cytoskeletal rearrangement and expression of different genes. The 'inside-out' direction, which involves signalling from the cytoplasm to the ECM, induces a conformational change to a high affinity binding state, as well as integrin clustering (Alon & Feigelson, 2002; Askari et al., 2009; Bökel & Brown, 2002; Ginsberg et al., 2005; Hynes, 2002). The 'inside-out' signalling leads to the increased affinity of integrins for ECM components, such as FN. α5β1 and αv-containing integrins are major FN-binding integrins, and disruption of the α5 gene in mice arrests somitogenesis after 10-12 somites have been

formed (Goh et al., 1997; Yang et al., 1993). Mice lacking α5 and αv integrins lack PSM segmentation (Yang et al., 1999).

Fibronectins are large dimeric glycoproteins comprised of types I, II and III modules. FN assembly requires α5β1 integrin, and FN cross-linking requires the application of tension from the actin cytoskeleton through α5β1. This stretches FN dimers and therefore reveals FN binding sites (Larsen et al., 2006; Mao & Schwarzbauer, 2005). FN expression is required for somitogenesis, and its loss disrupts somitogenesis in mouse (George et al., 1993), zebrafish (Jülich et al., 2005; Koshida et al., 2005), and xenopus (Kragtorp & Miller, 2007) embryos. Introducing a point mutation in the α5β1-binding motif gives the same phenotype as a α5β1-/- mouse, i.e. somitogenesis is halted after roughly 13 somite pairs are formed, and mutated FN fibrils are assembled through αv integrin binding to a novel FN binding site (Takahashi et al., 2007). α5β1-mediated adhesion to FN in PSM cells regulates cell-to-cell communication and membrane protrusion dynamics, which in turn are required for segmentation and elongation of the body axis (Girós et al., 2011).

During somite border formation, cells at the boundary undergo MET, and assemble a FN matrix along the boundary (Crawford et al., 2003; Holley, 2007; Larsen et al., 2006). The PSM is coated in a FN matrix: FN alignment is regulated by non-canonical Wnt signalling, but how is matrix formation restricted to certain tissues (Davidson et al., 2004; Goto et al., 1998)? The FN matrix is at first intermittent along the somite border, but as somite maturation progresses, the FN matrix becomes increasingly fibrillar until it is finally supplanted by a laminin matrix (Crawford et al., 2003). It has been recently shown that α5β1 clustering along the nascent somite border is independent of FN binding and precedes matrix formation. Ephrin reverse signalling is also sufficient to enable clustering. α5β1 is

expressed in adjacent cells, and reciprocally inhibits the formation of ECM and integrin clustering prior to activation. Upon activation, a self-organising mechanism exists to assemble the ECM on the tissue surface. It has been concluded, therefore, that Eph/ephrin signalling, ligand independent clustering, and reciprocal integrin inhibition restricts formation of the ECM to developing somites (Jülich et al., 2009).

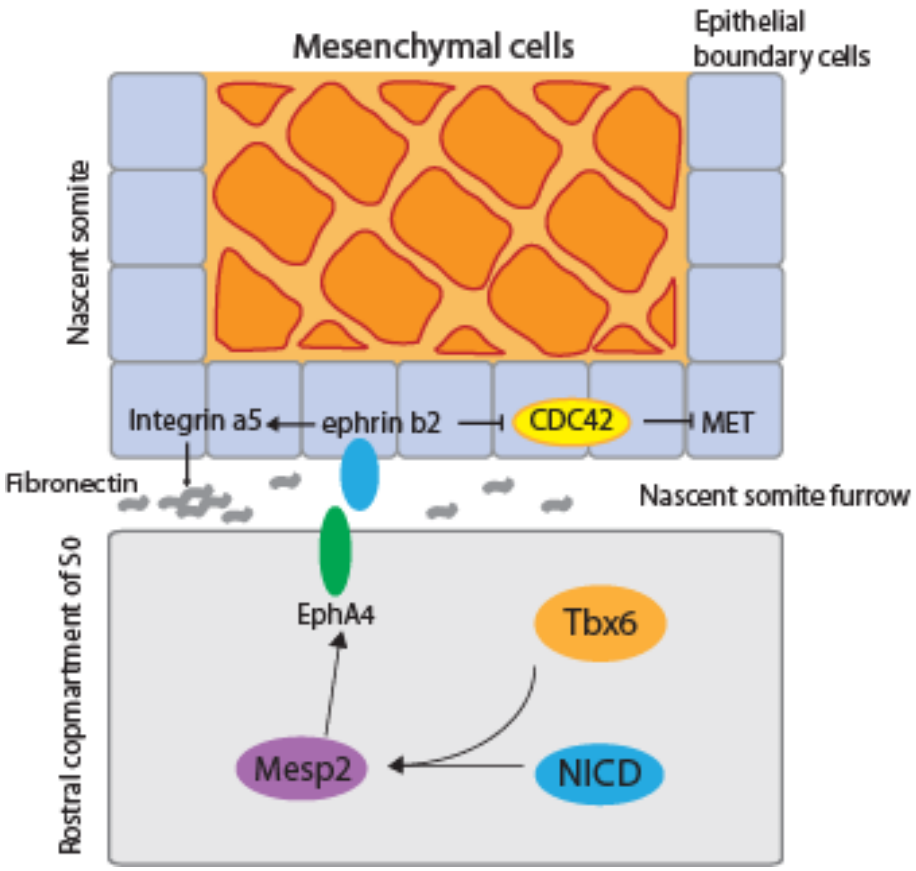


Figure J: The MET transition during somitogenesis

In cells at the anterior tip of the PSM, where the new somite boundary is formed, Mesp2 induces Eph-ephrin signalling activity, which in turn causes the formation of a new furrow. This process involves integrin α5 clustering at the somite boundary, which then recruits fibronectin-based ECM to the forming border. The induction of Eph-ephrin signalling also causes the epithelialisation of the somite boundary cells (adapted from Maroto et al., 2012).

13. Somite Differentiation

As somites mature, the cells become specified based on their location within the somite: the sclerotome is formed from the ventral-medial cells of the somite which undergo mitosis and become mesenchymal once more. These cells will eventually form chondrocytes (cartilage cells) and part of each rib. The remaining epithelial cells are arranged into three regions, as has been demonstrated by various fate mapping experiments with chick-quail chimeras (Brand-Saberi et al., 1998; Ordahl & Le Douarin, 1992). Cells in the two lateral portions of the epithelium form the myotome, a muscle forming region which then divides to form a second layer of muscle progenitor cells called myoblasts. This double layer of cells is known as the dermamyotome. Myoblasts formed in the region which is closest to the neural tube form the epaxial muscles, while those myoblasts furthest from the neural tube form the hypaxial muscles (Christ & Ordahl, 1995; Venters et al., 1999). The dermatome, which is the central region of the dorsal layer of the dermamyotome, generates the dermis (Figure K).

An important study used tissue explant culture with the chick embryo showed that signals from the dorsal neural tube, ventral neural tube floor plate (floor plate) and notochord are required for the expression of Myogenic differentiation 1 (MyoD), Myogenic factor 5 (Myf5) and myogenin in the somite (Munsterberg et al., 1995). In organ culture at HH11, myogenesis was inducted in the three most recently formed somites by the neural tube (with floor plate) and notochord, while in the more rostral somites, the dorsal neural tube alone is sufficient. Recombination of somites and neural tube from different axial levels of the embryo revealed that another signal is required to allow somite cells to be able to respond to inducing signals from the neural tube. Therefore, one signal from the

notochord/floor plate and another from the dorsal neural tube are required for myogenesis to occur (Münsterberg & Lassar, 1995).

It was subsequently shown that the notochord and floor plate secrete Sonic Hedgehog (Shh), and this ventral midline expression is required for induction and maintenance of the sclerotome (Borycki et al., 1998; Munsterberg et al., 1995); Wnt4 and Wnt6 that are secreted from the surface ectoderm are required for the induction of the lateral dermamyotome (Dietrich et al., 1997; Fan et al., 1997); and the myotome is induced by the expression of Shh from the notochord and floor plate, and sustained by the expression of Wnt1 and Wnt3a from the dorsal neural tube (Dietrich et al., 1997; Munsterberg et al., 1995). It is thought that the induction of these somite derivatives is concentration-dependent, and therefore the dorsal-ventral patterning of somites is regulated by a balance of dorsalising and ventralising signals, and the middle region (myotome) requires intermediate amounts of both signals (Dietrich et al., 1997; Munsterberg et al., 1995; Münsterberg & Lassar, 1995).

Myotome cells, which express MyoD and Myf5, eventually form the epaxial and hypaxial muscles, as well as part of the dermis of the back (Yusuf & Brand-Saberi, 2006). Notochord-derived Shh inhibits BMP4 from the lateral plate mesoderm extending to medial and ventral regions, thus preventing the conversion of sclerotome to muscle (Watanabe, et al., 1998). Noggin produced in the medial myotome also inhibits BMP4 from generating hypaxial muscle characteristics in these cells (Marcelle et al., 1997; McMahon et al., 1998).

Myotome cells are induced to differentiate into muscle cells by the expression of MyoD protein (Maroto et al., 1997; Pownall et al., 2002; Tajbakhsh et al., 1997). However, there are different mechanisms of muscle specification between the hypaxial and epaxial lineages.

In the lateral dermamyotome of the mouse embryo Pax3 is induced by Wnt proteins from the epidermis, as well as BMP4 and FGF5 from the LPM (Cossu et al., 1996). Following this induction, Pax3 then itself induces the expression of MyoD. Conversely, in the medial myotome, which eventually forms the hypaxial muscles, the protein Myf5 induces the expression of MyoD. MyoD and Myf5 are both myogenic bHLH transcription factors, and MyoD directly activates the expression of muscle-specific enzyme creatin phosphokinase (Lassar et al., 1989). It also positively regulates its own expression; so that once the MyoD gene is activated it will remain active thereafter. Myf5 and MyoD are specific to muscle cells types, and cells which express Myf5 and MyoD are therefore committed to becoming muscle cells; this has been proven in several assays where the transfection of these genes into a variety of cell types converts them into muscle cells (Thayer et al., 1989; Weintraub et al., 1989).

It has been shown that by down regulating of the transcription factor Pitx2, the number of premyogenic cells in somite is decreased; whereas overexpression of Pitx2 increases the myocyte number in somites, particularly in the epaxial region. Since Pitx2 is a target of the canonical Wnt/lef1 signalling pathway (Kioussi et al., 2002), it is though that Wnt signalling regulates the expression of Pitx2 to induce epaxial myogenesis in the somite (Abu-Elmagd et al., 2010).

In avian and murine embryos, Pax3 is expressed in the dorsal region of somites and the dermamyotome, and has been shown to have a key role in muscle regulatory functions. In Pax3-/- mouse embryos, the hypaxial and limb precursors do not migrate to their sites of myogenesis, but instead undergo apoptosis in the dermamyotome; in mice which are null for both Pax3 and Myf5, no myotome muscles are formed at all (Tajbakhsh et al., 1997). It is

thought that myogenesis does not occur in these embryos because Pax3 is required to prevent excessive apoptosis in the dermamyotome, and that Myf5 is required for the induction of MyoD in dorsal medial lip (DML) and ventral lateral lip (VLL) precursors. It has been demonstrated that Pax3 may also induce MyoD expression in myotome progenitor cells, as Pax3 misexpression in cultured PSM cells and embryonic neural tube induces MyoD expression and subsequent myogenesis (Maroto et al., 1997).

The dermamyotome differentiates in response to neutrophin3 (NT3) and Wnt1, both of which are secreted by the neural tube, and allows the dermatome to remain epithelial. By neutralising endogenous NT3, cells between the myotome and ectoderm become tightly aggregated, instead of forming loose dermal mesenchyme, showing NT3 to be crucial for dermatome dissociation (Brill et al., 1995).

Sclerotome formation is characterised by the EMT in ventral halves of the somites, and the migration of mesenchymal cells induced by factors secreted by the notochord (Christ et al., 2007). Cells extend filapodia and leave the epithelium (Trelstad, 1977). The maintenance of the epithelial structure of the dorsal somite region depends on Wnt6 expression in the overlying ectoderm (Linker et al., 2005). The DML and VLL of the dermamyotome are maintained by Wnt6 from the ectoderm and Wnt11 from the dermamyotome respectively (Geetha-Loganathan et al., 2006; Schubert et al., 2002). The central dermamyotome undergoes MET to eventually form the muscle and dermis (Geetha-Loganathan et al., 2006). The EMT and migration of mesenchymal cells in the sclerotome is accompanied by a down regulation of N-cadherin, which induces cell motility (Duband et al., 1987; Hatta et al., 1987; Takeichi, 1988; Šošić et al., 1997).

The sclerotome is also characterised by Pax (paired-box)-1 and -9 expression, which are induced by high levels of Shh (Dietrich et al., 1993; Koseki et al., 1993; Peters et al., 1995). Pax1 and Pax9 are required for the formation of cartilage, which in turn is required for the formation of vertebrae (Smith & Tuan, 1995). These cells also prevent muscle formation by expressing I-mf, an inhibitor of myogenic bHLH transcription factors that initiate muscle formation (Chen et al., 1996). Subsequently, Pax1; Pax9 -/- mice have shown that both genes are required for chondrogenic differentiation of sclerotomal cells (Rodrigo, 2003).

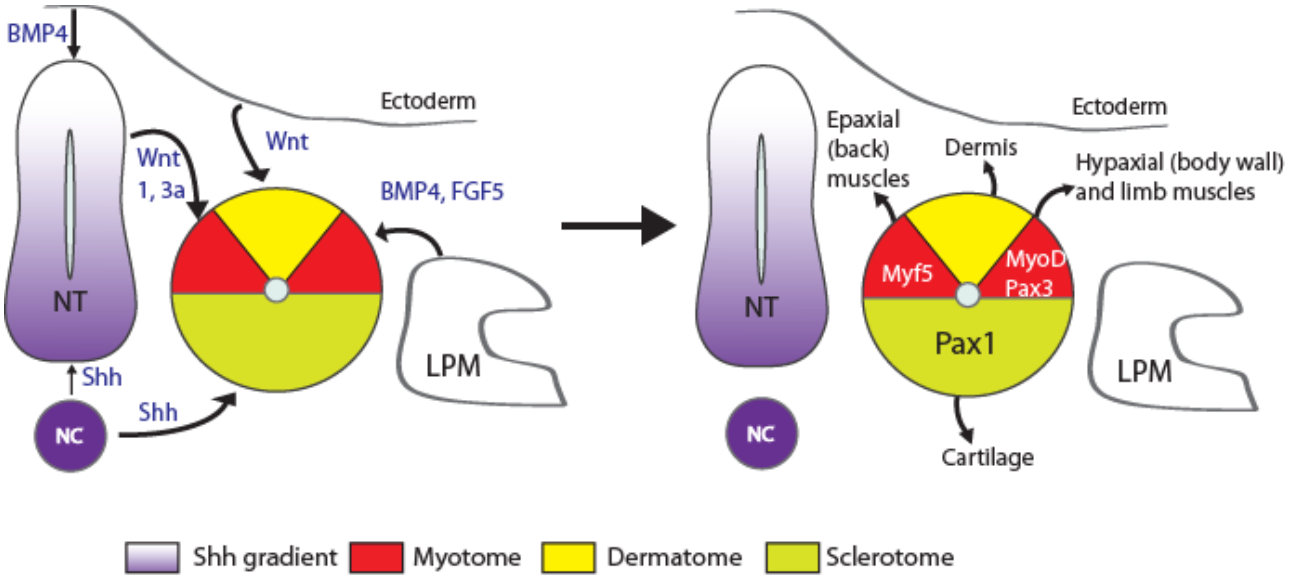


Figure K: Somite patterning

Wnt1 and Wnt3a, which are induced by BMP4 in the neural tube (NT), as well as low concentrations of Shh from the neural tube and notochord (NC), induce the formation of the epaxial myotome, which then expresses Myf5 and eventually forms the back muscles. Wnt proteins from the epidermis, along with BMP4 and FGF5 from the lateral plate mesoderm (LPM), induce the hypaxial myotome, which then express Pax3 and MyoD and eventually form the body wall and limb muscles. NT3 and Wnt1 specify the dermamyotome, which eventually forms the dermis. High concentrations of Shh from the notochord specify the sclerotome, which induces it to express Pax1 (adapted from Maroto et al., 2012).

14. Anterior to Posterior Specification

As mentioned above, *Hox* genes are responsible for patterning the axial and paraxial tissues in vertebrate embryos. All axial and paraxial structures between the mid-hidbrain and the tip of the tail acquire a specific identity through a unique combination of *Hox* gene expressions, known as the 'Hox code' (Figure L) (Kessel & Gruss, 1991). Thus, for example somitic segments in the thorax develop into ribs while those more caudally develop into lumbar vertebrae and this is largely due to the Hox code they express.

In order to understand how the code is established, there have been three methods of experimental analysis: gene targeting, retinoic acid teratogenesis, and comparative anatomy.

14.1 Gene targeting

Studies which have looked at the effect of *Hox* gene mutations and deletions have provided the most information as to how the Hox code is established. *Hoxa3*-/- mice die soon after birth, revealing that the neck cartilage is abnormally short and thick, whereas *Hoxa2*-/- mice show similar defects to the *Hoxa3*-/- mice, except the deficiencies are more anteriorly located (Chisaka & Capecchi, 1991; Gendron-Maguire et al., 1993). Regionally specific organs fail to form in these mice as well, indicating that *Hox* genes are also required for their production. Some region-specific organs are respecified and some skeletal segments resemble more anterior segments as a result, such as the case of the Hoxc8-/- mouse (Le Mouellic et al., 1992). When more than one *Hox* gene is knocked out, this can have severe consequences. In the case of the Hoxd3-/- mouse, the first cervical vertebrae had mild

abnormalities, and in the Hoxa3-/- mouse, there are no defects of this bone. However, in a Hoxa3;Hoxd3-/- mouse, there is no cervical vertebra, indicating that in some functions, one *Hox* gene paralogue can substitute for another (Condie & Capecchi, 1994).

14.2 Retinoic acid teratogenesis

When RA is overexpressed, the *Hox* genes are activated in the wrong locations along the anterior-posterior axis. Some *Hox* genes have RA receptor sites in their enhancers, which indicates that an up regulation of RA causes their expression in cells which would not normally express them (Conlon & Rossant, 1992). In embryos exposed to RA at E8, the first one or two lumbar vertebrae were transformed to thoracic vertebrae, and in some cases the posterior regions failed to develop (Kessel & Gruss, 1991). The Hox codes were also shifted anteriorly, and when the posterior genes were not expressed, the caudal part of the embryo failed to form.

14.3 Comparative anatomy

Several studies have shown that by comparing the vertebrae of different species, it is possible to predict the type of vertebra formed based on the constellation of *Hox* gene expression. An example of this is the observation that the border between cervical and thoracic vertebrae occurs at vertebra position 7-8 in the mouse, and 14-15 in the chick. In both cases, *Hox5* paralogues are expressed in the last cervical vertebra, while *Hox6* paralogues are expressed in the first thoracic vertebra.

14.4 Notch, Wnt and FGF pathways in Hox regulation

Wnt and FGF signalling have been implicated in the regulation of *Hox* gene expression, as well as regulating primitive streak emergence and elongation (Ciruna & Rossant, 2001; Forlani, 2003). In Wnt3a and Fgfr1 hypomorphic mutants, *Hox* gene expression domains become restricted to more posterior regions, indicating that FGF and Wnt proteins either directly or indirectly affect *Hox* gene expression (Ikeya & Takada, 2001; Partanen et al., 1998).

RA distribution in the PSM is also likely to be crucial for *Hox* gene regulation, as misexpression leads to defects in anterior-posterior patterning and *Hox* gene regulation, as well as mesodermal patterning (Abu-Abed, 2001; Niederreither et al., 1999; Sakai, 2001). However, the function of RA in this context is hard to prove, as there is still *Hox* gene expression in RA-impaired embryos (Niederreither et al., 1999).

Notch signalling is also thought to be involved in this process: loss-of-function and gain-of-function experiments which alter the periodicity of Notch activity have shown effects on Hox gene expression in the PSM and in subsequent vertebral patterning (Cordes et al., 2004; Zákány et al., 2004). This Notch activity could be linked with the effects of Wnt signalling, as it is known that there is some degree of regulation of Notch activity by the Wnt pathway (Galceran et al., 2004; Hofmann et al., 2004). In a study where the *Hoxd* cluster was replaced with LacZ under the Hox promoter this resulted in bursts of expression in phase with the segmentation process, indicating that *Hox* genes are activated each time the cells approach the somite boundary (Zákány et al., 2004). In mice double-null for the Notch ligand *Dll1*, which have impaired Notch signalling, there is an anterior transformation of vertebral identity caused by a posterior shift of several Hox gene domains (Cordes et al.,

2004). In *lfng*-/- mice, there was a similar phenotype, which indicates that altering the cyclic expression or impairing of Notch activity causes a delay in *Hox* gene activation (Cordes et al., 2004). However, in mice which have a complete lack of Notch activity (RBPj-/-), this causes a drastic effect on *Hox* gene activation by abolishing the expression of mesodermal *Hox* genes (Zákány et al. 2004), which clearly implicates Notch signalling in the regulation of *Hox* gene expression.

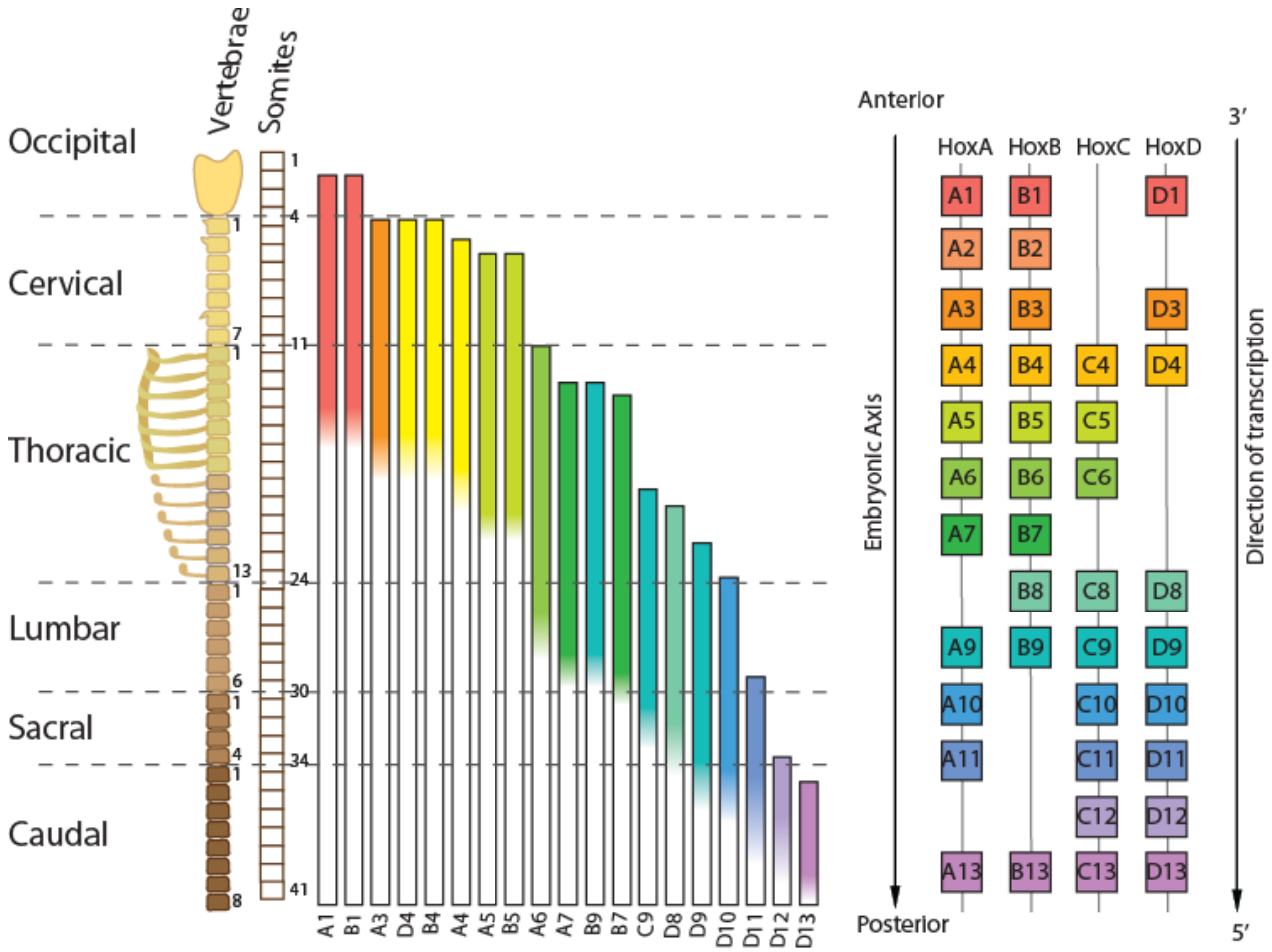


Figure L: Anterior-posterior identity is determined by *Hox* genes

Hox genes play an important role in specifying the anterior-posterior body plan during development, and are found in ordered clusters in the genome. In vertebrates there are 4 clusters (A, B, C, and D), each with multiple *Hox* genes, and different combinations of *Hox* genes specifies each vertebra with a distinct morphology, depending on its location in the anterior-posterior body axis (adapted from Maroto et al., 2012).

15. MicroRNAs and Somitogenesis

It has been shown that mRNA and protein instability is crucial for the oscillations of the segmentation clock and therefore somitogenesis. This was demonstrated by prolonging the half-life of Hes7 protein by 8 minutes, which disrupted somitogenesis in the mouse embryo (Hirata et al., 2002). One means by which the stability of mRNAs and proteins could be regulated is through the action of microRNAs (miRNAs). MiRNAs are a class of short (19-25 nucleotide (nt)), single-stranded RNA molecules which exist in both plants and animals. They are transcribed primarily by RNA Pol II, as primary (pri-) miRNAs, which are then cleaved in the nucleus by Drosha, a RNase III enzyme, which produces a shorter (roughly 65 nt) precursor (pre-) miRNA hairpin structure. Once exported to the cytoplasm, another RNase III enzyme called Dicer further processes the miRNA, producing a mature 19-25nt structure. This is then incorporated into a miRNA-induced silencing complex (RISC), which also contains an Argonaute (AGO) protein (in flies and mammals the main enzyme for this is AGO2 (Liu et al., 2004; Okamura et al., 2004), along with other factors, and acts as the effector of the miRNA pathway. There are two possible models for the mechanism of miRNA-mediated gene regulation: translation of mRNAs is stopped at the initiation stage, or translation is inhibited following initiation (Figure M). Evidence for the former argument is that in cell culture, AGO2 binds to a m7G cap of mRNA through the same binding domain that elF4E (translation initiation factor) uses, therefore they competitively inhibit each other and RISC-binding occurs instead of translation initiation (Mathonnet et al., 2007). Evidence for the second argument is that mRNAs being inhibited by miRNAs are attached to actively translating ribosomes, indicating that they do not inhibit the initiation of translation (Nottrott et al., 2006). This mechanism of inhibition could work by rapid degradation of

protein product, or by incomplete protein production due to ribosome dissociation. Another method of inhibition is for miRNAs causing target mRNAs to be degraded, as is shown by co-localisation of RISC and mRNA degradation factors in P-bodies (processing bodies), cytoplasmic foci that are known sites of mRNA degradation (Valencia-Sanchez, Liu, Hannon, & Parker, 2006). The sequestration of mRNAs in P-bodies following blocked translation is possible, as P-body component GW182 is shown to directly interact with AGO2 and is recruited to the mRNA (Behm-Ansmant et al., 2006; Wakiyama et al., 2007). The precise action of miRNAs has been shown to be immensely important to the regulation of numerous processes during development, such as the proliferation of cells, apoptosis and differentiation (Goljanek-Whysall et al., 2011; Spruce et al., 2010; Sweetman et al., 2006).

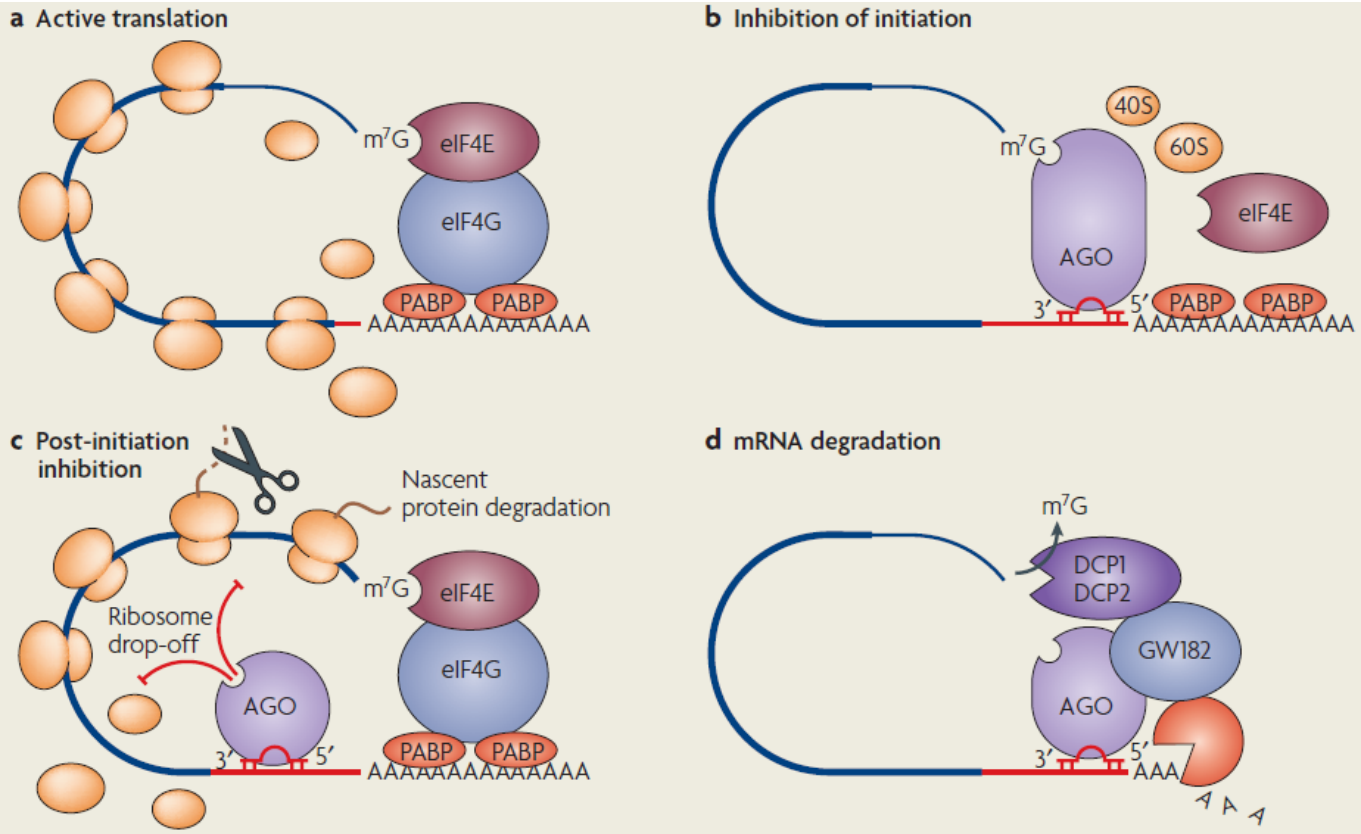


Figure M: Different mechanisms of miRNA-mediated gene regulation

The mechanism of how miRNA-mediated gene regulation affects translation (A) is currently unknown. One main view is that the translation of mRNAs is inhibited at the point of initiation (B), while the other main view is that inhibition occurs post-initiation (C). The variable level of target mRNA degradation (D) observed adds another layer of uncertainty to this process: sequestration of target mRNAs could be a step which follows the blocking of translation, or a causative event in miRNA degradation.

15.1 MiRNA regulation of Notch and Wnt pathways

Two signalling pathways which are crucial for somitogenesis, Notch and Wnt, are regulated by miRNAs in tissues other than the PSM. Functional assays have shown that the miRNA miR34a binds to the 3' untranslated regions of Notch1 and ligand Jagged1, and that the ectopic expression of miR-34a inhibits Jagged1 and Notch1 expression, reducing the capacity of cervical cancer cell lines to become invasive. This reduced invasion is nullified by transfecting the cells with NICD, indicating that miR-34a is inhibiting Notch signalling, which in turn is affecting invasion capacity of these cells (Pang et al., 2010). MiR-236 was identified by microarray analysis in glioma tumour stem cells after siRNA-knockdown of Notch1 as a potential mediator of Notch signalling in these cells. They further show that miR-236 inhibits the Notch pathway, and is in turn inhibited by Notch, indicating the presence of a double negative feedback loop. In the xenopus embryo, the Notch target hairy1 is the single key target that mediates the effects of miR-9 on neurogenesis, proliferation, and apoptosis in the fore- and hindbrain. Hairy1 target-protector experiments produced the same effects as the miR-9 MO phenotype *in vivo* (Bonev et al., 2011). In the zebrafish, several FGF components and Her5 have been identified as possible targets of miR-9 involved in mid-hindbrain formation (Leucht et al., 2008). MiR-1, -25, and -613 were shown to be inhibitors of the Wnt pathway by epistatis-based functional assays in human HEK293 cells (Anton et al., 2011). These experiments have shown that miR-25 is likely to function at the level of β-catenin, with miR-1 and -613 acting upstream of β-catenin. Based on this evidence, it is possible that miRNAs may play an important role in somitogenesis. Theoretical models have been designed in an attempt to explain the dynamic nature of the miRNA mechanism in somitogenesis. In one such model, small RNAs (sRNAs) were incorporated into the gene

regulation process. This model predicts that defective miRNA expression leads to skeletal defects, which has been supported by data showing that interfering with miRNA expression caused formation of different numbers of vertebrae. In these zebrafish experiments, overexpression of miR-196 caused (among other things) a reduction in the number of somites, whereas loss-of-function studies showed an increase in somites (He et al., 2011), which further indicates that miRNA function may well be involved in setting the pace of somitogenesis. However, despite recent discoveries detailing the phenotypic defects of miRNA-deficient organisms, a mechanism for miRNAs in somitogenesis has not yet been identified.

Project aims

Somitogenesis is crucial for the development of a segmented body axis in vertebrate species. The presence of a segmentation clock in the PSM which drives the oscillations of cyclic clock genes is thought to underlie somitogenesis. Components of the Notch, Wnt and FGF pathways are known to oscillate in the mouse PSM, although the level of cross-talk between these pathways is currently poorly understood.

The main aim of this thesis is to firstly characterise the expression profiles of the Notch pathway ligands in both the chick and mouse embryos, and to subsequently investigate how their transcription is regulated, in particular to determine whether the Wnt signalling pathway is required, and will determine whether the Dll ligands act as a point of cross talk between these two pathways.

The second aim of my project is to ask whether the process of somitogenesis can occur in the absence of oscillations of clock genes in the PSM of both chick and mouse models.

Finally, I will begin to ask how the lack of miRNA activity in the PSM affects the process of somitogenesis. For this I will make use of a recently available mouse model which lacks the miRNA-processing enzyme Dicer, in order to look at how the skeletons of these mice are affected, and whether the segmentation clock is disrupted in the PSMs of these embryos.

Material and Methods

Molecular Biology

Transformation

The plasmids containing the sequences for making anti-sense RNA probes were transfected into DH5α *E. coli* cells. A 50 µl aliquot of the bacterial cells was thawed on ice for 15 minutes. 2 µl plasmid solution was added to 50µl cells on ice, then mixed and left to incubate for 30 minutes. After this period, the bacteria were heat-shocked at 37°C for 60 seconds, and then incubated on ice for 5 minutes. Then 0.25 ml S.O.C. media was added to the cells, and incubated at 37°C with slight shaking for 30 minutes. Following this incubation period, 20 µl bacterial cells were then spread on an agar plate containing the appropriate amount of antibiotic. The plate was then incubated overnight at 37°C.

Maxiprep

Once the overnight cultures had developed, one colony was incubated in 10 ml LB media with antibiotic for 8 hours on a 37°C rocker. Once this incubation was over, the culture media was added to 240 ml LB media at 37°C, and left on the rocker overnight. The next day, the culture media was spun down in a centrifuge at 6,000 rpm for 15 minutes at 4°C, generating a bacterial pellet. The supernatant was then discarded, and the maxiprep protocol was undertaken according to the Qiagen protocol. The plasmid DNA stock solution were then stored at -20°C.

Anti-sense RNA Probe Synthesis

Plasmid DNA (10 µg) containing a fragment of the target gene was first linearised with the corresponding restriction endonuclease enzyme. Following purification with the Qiagen PCR purification kit, the DNA was eluted in 50µl water, and 2µl of linearised DNA was analysed by electrophoresis using a 1% agarose gel.

Linearised DNA (1 µg) was then subjected to *in vitro* transcription (1x Transcription buffer (Promega), 10mM DTT, 10% digoxigenin labelled (or fluorescein labelled) NTPs, 10% RNA polymerase, 5% RNase inhibitor (Roche)) at 37°C for 3 hours to make anti-sense RNA probes (see Table 1 for a complete list of probes generated). After 3 hours, DNase I was added to remove any remaining DNA from the mixture, and left for one more hour at 37°C. At this point the RNA was purified using the Qiagen RNA mini kit and eluted in 100 µl water (RNase-free), allowing for a final RNA concentration of 10 µg/ml. Confirmation of *in vitro* transcription was shown by analysing 5 µl RNA probe by electrophoresis using a 1% agarose gel. Following this, the RNA probe was stored at -20°C.

Table 1: Anti-sense RNA probes (exonic and intronic) used for *in situ* hybridisation

Probe	Enzyme	Polymerase	Origin of plasmid DNA
cDll-1(exonic)	EcoR I	T3	D. Henrique lab
cDll-1(intronic)	Sac II	SP6	M. Maroto
cUncx4.1(exonic)	Sac II	SP6	M. Maroto
mAxin2(exonic)	Sal I	T7	B. Herrmann lab
mDll-1(exonic)	Xho I	T3	Larue et al. 1996
mDll-1(intronic)	BamH I	T7	Z. Ferjentsik
mDll-3(exonic)	Xho I	SP6	Z. Ferjentsik
mDll-3(intronic)	BamH I	T7	Z. Ferjentsik
mHes7(exonic)	Spe I	T7	R. Kageyama lab
mHes7(intronic)	Spe I	T7	R. Kageyama lab
mLfng(intronic)	Hind III	T3	D. Ish-Horowitz lab
mSnail1(exonic)	Sal I	T7	Nieto lab
mSnail1(intronic)	Sal I	T7	R. Bone
mTbx6(intronic)	Spe I	T7	M. Schimpl
mUncx4.1(exonic)	Xho I	T7	P. Gruss lab

Embryo Manipulation Techniques

Chick embryo dissection

White leg horn *gallus gallus* eggs were sourced from the Winter farm in Royston. To develop the embryos, the eggs were incubated in a humidified 38.5°C incubator until the correct stage of development, according to the Hamburger Hamilton developmental table (Hamburger and Hamilton, 1992), was reached. At this point, the embryos were dissected into PBS with 2mM EGTA (unless the tissue is to be cultured, in which case the embryos were dissected in PBS), and much of the extra-embryonic tissues were removed. The whole embryos were then placed in fixative using a dissection strainer spatula to keep the embryos as flat as possible. The embryos were then fixed in 4% formaldehyde in PBS/EGTA at room temperature for 2 hours or overnight at 4°C, followed by two washes for 10 minutes in PBS-0.1% Tween 20 (PBST), then one wash in 50% ethanol in PBST for 5 to 10 minutes, and two washes in 100% ethanol for 5 to 10 minutes. At this point the embryos were stored at -20°C.

In ovo drug incubation

Eggs were incubated for 17 hours up to HH4, then the tops of the egg shells were removed and excess albumen was removed (not too much, the volumes of albumen eggs were similar to prevent large differences in concentration). 300 µl of LY-411575 (10 µM) or the equivalent volume of DMSO solution was added onto the very edge of the embryo, so as to avoid mechanical damage to the embryo. The eggs were then sealed in order to prevent them from dehydrating in the incubator: this was done by covering the top with electric tape, ensuring there is a tight seal around the shell. The eggs were then placed in the

incubator and left to develop overnight. 12 hours after the initial drug addition, at which the embryos had developed to around HH7/8, the tape was removed from the tops of the eggs, and another 300 µl of LY or DMSO was added to the embryos in the same manner as before, before the eggs were again sealed and placed in the incubator for 6 hours. After this incubation, the embryos had reached roughly stage HH9/10, and were subsequently dissected into PBS with 2mM EGTA, and fixed in 4% formaldehyde in PBS/EGTA for 2 hours at room temperature, before dehydration (see above) and storage at -20°C. All incubation took place at 38.5°C in a humidified incubator. Solutions were kept on ice (±0°C) whilst in use, and stored at 4°C in the fridge until required for use (used within 5-6 hours of making up).

In ovo electroporation

pCIG and pCIG.NICD constructs (Dale et al., 2003) were made up to a concentration of 0.5 µg/µl along with Fast Green solution (Sigma) in distilled water. The plasmid solutions were then electroporated to the PSM precursor cells in the primitive streak of HH4 embryos using a BTX electroporator with the following conditions: 6 pulses of 50 ms pulse length at an interval of 150 ms at a voltage of 10 V. Following electroporation, the embryos were incubated at 38.5°C in a humidified incubator for 20 to 24 hours. Expression in the PSM and somites of the embryos were observed with the Hamamatsu camera, before the embryos were fixed for the in situ hybridisation protocol.

Preparation of agarose blocks and Cryosectioning

Following the fix step, embryos were washed twice for 10 minutes in PBS, followed by overnight incubation in 30% sucrose in PBS. Once the embryos had sunk to the bottom of the plate wells, the embryos were placed into agar blocks (1.5% LB agar, 3% sucrose in PBS) and positioned appropriately until the agar had set. Following this, the agar blocks were trimmed into a pyramid shape and left to equilibrate in 30% sucrose in PBS overnight at 4°C. Once the blocks had sunk, they were frozen on dry ice and wrapped in tin foil, which was stored at -80°C until sectioning. Cryosectioning took place in a cryostat, which took transverse sections (at roughly 16 μm) through the block, and the sections were collected on Superfrost slides and stored at -20°C.

Mouse Embryo Dissection

E9.5 or E10.5 embryos were harvested from CD-1 mice; the uterus was removed from the mother, and placed into autoclaved PBS. The embryos were removed from the uterine horns into PBS, the reichardt membranes were removed from the embryos, and the tails were then removed from the rest of the embryos. At this point the tails were fixed and dehydrated in the same manner as wholemount chicken embryos.

Half PSM explant generation

Once the tails were removed from the embryos in PBS, they were transferred as quickly as possible into culture media (DMEM-F12 + 0.1% Glutamax (GIBCO) supplemented with 10%

FBS, 1% pencillin/streptomycin, and 10 ng/ml bFGF (Peprotech)). The tails were cut so that between 5 and 10 somites remained, and positioned so that the neural tube was facing upwards. The tungsten needle was used to bisect the tail, generating two identical explants. The explants were then transported into the lid of a 35 mm plate in a drop of media (either both explants together or in separate lids), before flipping the lid over so that a "hanging drop" of media is created with the explants inside them. The plates were then placed inside a wet tray, and then into a 38.5°C humidified incubator with 5% CO_2. Following incubation, the samples were then placed into the same fix used for whole tails, and were fixed, dehydrated and stored in the same way.

Fix and Culture

For this technique, one explant of an embryo would be cultured for 30 to 45 minutes before fixing, while the other explant would be kept in culture for another 60 minutes longer (i.e. half a cycle) before fixing. This technique allows the observation of dynamic gene expression within the PSM of one embryo.

Culture and Culture

Explants would be cultured for 30 to 45 minutes before fixing both sets at the same time. This would allow us to observe the oscillation of certain cyclic genes relative to others in the same embryo.

Drug Culture

One explant was cultured in media containing a pathway-inhibiting drug, and the other explant would be cultured in media containing the equivalent volume of the drug solvent (e.g. DMSO), but without the drug. Both explants would be cultured for the same amount of time (usually 3-4 hours), before being fixed at the same time.

To observe the level of apoptosis in the explants following drug treatment, NucView 488 Caspase-3 substrate (Biotium) was added to the media in the last hour of incubation at a concentration of 10 µg/ml. Upon the completion of the incubation, the explants were imaged with the Hamamatsu camera, and images were taken using Volocity 5.5 software before the samples were placed into fixative solution.

Preparation and Sectioning of Paraffin Blocks

The mouse tails were fixed in 4% paraformaldehyde in PBS, following dissection in PBS, for 2 hours at 4°C. The tails were then dehydrated in an ethanol series, followed by two 1 hour washes in xylene, and one 1 hour wash in paraffin at 60°C. The samples were then incubated overnight in paraffin at 60°C. Following this incubation, the samples were placed into blocks and cooled to room temperature.

The blocks were prepared for sectioning by cutting the paraffin wax to a pyramid shape. The block was then placed into a myotome and 7 µm sections were produced. Three or four sections were then placed in a 50°C water bath and collected on a Superfrost slide. The slides were then heated in a 60°C oven overnight to remove excess wax, followed by storing at room temperature.

Detection Techniques

WISH

The wholemount *in situ* hybridisation (WISH) protocol was performed as previously recorded (Henrique et al., 1995). The embryos were rehydrated from either ethanol or methanol, and washed twice in PBST before treating with 10µg/ml Proteinase K in PBST. Times of incubation varied according to the stage of development: for chicken embryos, the time of incubation was equal to the Hamburger Hamilton stage number (e.g. HH10 = 10 minutes); for mouse embryos, E9.5 embryos were treated for 10 minutes, and E10.5 embryos were treated for 15 minutes (explants were treated for 5 minutes). Following Proteinase K treatment, the embryos were rinsed briefly in PBST, then post-fixed for 30 minutes in post-fixative solution (4% Formaldehyde, 0.1% Gluteraldehyde in PBST). After this step the embryos were then washed twice for 10 minutes in PBST, before a 10 minute wash in 50% hybridisation mix (50% formamide, 1.3x SSC, 5mM EDTA, 50µg/ml tRNA, 0.2% Tween20, 0.1% SDS, 100µg/ml heparin) in PBST at 65°C. The samples were then rinsed once in Hybridisation mix and incubated in Hybridisation mix for a minimum of 2 hours at 65°C. Following this incubation, the Hybridisation mix was removed and replaced with 20 µl/ml anti-sense RNA probe in hybridisation mix, and the samples were incubated at 65°C overnight.

After the overnight incubation, the probe was recovered and stored at -20°C. The samples were then rinsed twice in pre-warmed hybridisation mix and then washed for three 1 hour periods in Hybridisation mix at 65°C. Following this, the samples were then washed in 50% Hybridisation mix in TBS-0.1% Tween 20 (TBST) at 65°C for 15 minutes. The samples were rinsed twice in TBST at room temperature and then washed in TBST for 30 minutes. After

this wash, the samples were then pre-incubated in 2 ml of blocking solution (2% Blocking Buffer Reagent, 20% heat-treated goat serum in TBST) for a minimum of 2 hours. Following this pre-incubation, the samples were incubated in 1:2000 α-DIG-Alkaline phosphatase antibody in blocking solution overnight at 4°C.

After this incubation, the samples were rinsed three times in TBST, and then washed in three 1 hour periods in TBST. This was followed by two 10 minute washed in NTMT (100 mM NaCl, 100mM Tris-HCl (pH 9.5), 1% Tween20, 50 mM $MgCl_2$), then incubation in colour reagent (0.27% 50 mg/ml NBT (Promega), 0.14% 50mg/ml BCIP (Promega) in NTMT) at 37°C in the dark. Once the colour had developed sufficiently, the samples were washed twice in NTMT for 10 minutes, then three times in PBST for 5 minutes, followed by fixing for 2 hours at room temperature in 4% formaldehyde in PBST. The samples were then washed twice in PBST for 10 minutes and stored in 0.1% sodium azide in PBST at 4°C.

For the use of intronic probes, the following modifications were made: samples were incubated for 2 nights in a low stringency Hybridisation mix (50% formaldehyde, 5x SSC, 50 µg/ml tRNA, 0.2% Tween20, 0.1% SDS, 100 µg/ml heparin) and all post-hybridisation washes on the second day were in post-hybridisation buffer (50% formaldehyde, 0.1% Tween20, 1x SSC).

Double FISH

The double fluorescent *in situ* hybridisation (FISH) protocol is very similar to the wholemount *in situ* protocol, with some modifications. Samples were incubated with two anti-sense RNA probes: one labelled with DIG, the other labelled with fluorescein, both at

20 µl/ml in Hybridisation mix (the most robust probe was fluorescein labelled). At the antibody stage, the samples were incubated with 1:2000 α-F-Alkaline phosphatase overnight at 4°C. After the TBST washes on Day 3, the samples were washed twice for 10 minutes with 0.1M Tris (pH 8.0), followed by fluorescence detection with Fast Red solution (one Fast Red tablet (Roche) dissolved in 2 ml 0.1 M Tris-HCl (pH 8.2)) in the dark at 37°C. Once the fluorescent signal was sufficiently strong, the samples were reincubated in blocking solution at room temperature for a minimum of 2 hours, followed by incubation with 1:200 α-DIG-Horseradish peroxidase overnight at 4°C. The Day 3 TBST washes were repeated after this incubation, and then the embryos were washed with Amplification buffer (Perkin Elmer) for 1 minute, followed by incubation with TSA-Flourescein (Perkin Elmer, 1:50 in Amplification buffer) for 1 hour at room temperature in the dark. Following this incubation, the samples were washed three times for 5 minutes in TBST and then for 45 minutes in 1% H_2O_2 in TBST at room temperature to deactivate the HRP. This was then followed with three more washes for 5 minutes at TBST. The samples were then imaged before fixing.

LNA *in situ* hybridisation

Locked nucleic acid (LNA) probes were used to detect the presence of miRNAs in the mouse PSM. Prepared embryos were transferred to prehybridization solution (50% formamide, 53 SSC, 2% blocking powder, 0.1% Tween-20, 0.1% CHAPS, 50 mg/ml yeast RNA, 5 mM EDTA, 50 mg/ml heparin, and DEPC water). Prehybridizations and hybridizations were carried out 22°C below the predicted melting temperature of the LNAs, or at the indicated temperatures. LNA probes were added to 1 ml fresh prehyb buffer and hybridization carried

out for 48 h. Following hybridization, the embryos were transferred to wash solution containing 23 SSC, 0.1% CHAPS prewarmed to the hybridization temperature and washed three times for 20 minutes. This was followed by three times for 20 minute washes in 0.23 SSC, 0.1% CHAPS. Embryos were rinsed twice in KTBT (50 mM Tris at pH 7.5, 150 mM NaCl, 10 mM KCl, 1% Tween-20) and then incubated in 20% sheep serum in KTBT at 4°C for 2–4 hours. Anti-DIG antibody binding (1:2000) was carried overnight at 4°C. Following antibody incubation, embryos were washed five times for 1 h in KTBT. Embryos were then washed twice 10 min in NTMT. Colour reactions were carried out in NTMT containing NBT/BCIP at 1–6 hours at room temperature until signal or background became visible, followed by overnight washing in TBST. Embryos were then dehydrated through a graded methanol series to remove background and enhance signal and then rehydrated and stored in PBS plus 0.1% sodium azide.

Immunohistochemistry

After de-parafinisation and rehydration of the samples in an ethanol series, antigen was retrieved by boiling samples in a pressure cooker for 2 hours in sodium citrate (10 mM, pH 6.0) to avoid evaporation. Slides were allowed to cool to room temperature and were washed four times in distilled water for 3 minutes, and then PBS for 3 minutes. Endogenous peroxidase activity was quenched using 1% H2O2 in 100% methanol for 40 minutes at room temperature, followed by three washes in PBS for 5 minutes, and two washes in 0.3% Triton in PBS for 10 minutes. For blocking, samples were incubated for 1 hour in histoblock solution (3% bovine serum albumin (BSA), 20 mM $MgCl_2$, 0.3% Tween 20, 5% fetal bovine

serum in PBS) before incubation with primary antibody in histoblock solution at 4°C, overnight (see Table 2 for the list of antibodies used). This was followed by washing with PBS twice for 5 minutes, and 0.3% Triton X-100 in PBS twice for 5 minutes. Slides were then incubated with secondary antibody conjugated with HRP (1:100 in 5% BSA in PBS; Vector Laboratories) for 1 hour at room temperature, followed by two washes in PBS for 5 minutes and two washes in 0.3% Triton in PBS for 5 minutes. Finally, Tyramide-cynanine 3 (from the tyramide signal amplification (TSA)-cynanine 3 kit (Perkin-Elmer)) was added to the samples for 10 minutes at room temperature in the dark. Washes following amplification were done twice in 0.3% Triton in PBS for 5 minutes and nuclei counterstained using 4',6-diamidine-2-phenylidole-dihydrochloride (DAPI, Vector Labs). Slides were mounted In Prolong Gold and stored at -20°C.

Skeletal Preparations

E16.5 mouse embryos were obtained by caesarean section, eviscerated, and left in water at room temperature for 3-6 hours. This was followed by 1 minute incubation at 65°C, then the skin was removed and the embryos were fixed in 100% EtOH for 3 days. Once fixed, the embryos were then incubated in Alcian blue (150mg/L in 80% EtOH, 20% acetic acid) for 12 hours, and then washed in 100% EtOH for a further 12 hours. The embryos were then treated with 2% KOH for 6 hours and stained with Alizarin red (50 mg/L in 2% KOH). Following this staining, the embryos were then washed with 2% KOH and stored in 25% glycerol at 4°C.

Imaging

All *in situ* hybridised embryos were imaged using a Leica MZ16 APO microscope using a Jenoptik camera, and the images were recorded using Volocity 5.5 software. The FISH samples were imaged with a Hamamatsu camera.

Sections that were treated with anti-TBX6 antibody were also imaged using the Leica MZ16 APO microscope. Sections that were treated with anti-Notch1, anti-DLL1, and anti-NICD antibodies were analysed on a Zeiss 710 microscope and the images were recorded using Zeiss Efficient Navigation (ZEN) software.

Table 2: Antibodies used for Immunohistochemistry

Antibody	Species	1° concentration	Origin of antibody
anti-Notch1	Mouse	1:50	BD Biosciences Pharmingen™
anti-DLL1	Rat	1:50	E.Kremmer lab
anti-NICD	Rabbit	1:100	Cell Signalling Technology®
anti-TBX6	Rabbit	1:1000	Y. Saga lab

Results

Chapter 1: The Regulation of the Notch ligand *Dll-1* in the vertebrate PSM

Introduction

Vertebrate somitogenesis relies on a molecular oscillator acting in the unsegmented PSM driving periodic gene expression across the PSM with the same periodicity as somitogenesis. The genes that display this oscillatory expression profile belong to three different signalling pathways, namely Notch, FGF and Wnt. There is much evidence which indicates that there are multiple levels of cross-talk between Notch, Wnt and FGF signalling pathways within the PSM. Previous studies have highlighted the necessity of Wnt signalling, both indirectly (via Wnt downstream target TBX6) and directly (via Wnt transcription factors TCF/LEF1) upon the transcriptional expression of the Notch ligand *Dll1* in the mouse PSM (Galceran et al., 2004; Hofmann et al., 2004), thus acting as a point of cross talk between these two pathways. However, I wanted to further explore this relationship: to fully characterise the expression profiles of all the *Dll* mRNA transcripts in both the mouse and chick PSM; to analyse the extent to which Wnt signalling is required for their expression; and to determine the role of Notch signalling in their transcriptional activation.

In this chapter, different pharmacological inhibitors were used to abolish Notch and/or Wnt signalling in PSM explant culture. The drug which was used to inhibit Notch signalling was

the γ-secretase inhibitor: *N*2-[(2*S*)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl]-*N*1-[(7*S*)-5-methyl-6-oxo-6,7-dihydro-5*H*dibenzo[*b*,*d*]azepin-7-yl]-L-alaninamide (LY-411575). The selective inhibition of γ-secretase prevents the intracellular cleavage of NICD, and thus prevents the downstream transcription of Notch target genes. Previous studies in our lab have made use of another inhibitor DAPT, which was used at the higher concentration of 100μM, whereas LY411575 (LY) is found to be effective at a much lower concentration of 150nM, thus making it a more attractive inhibitor due to diminished expectations of secondary effects.

While it has been reported previously that Wnt signalling plays a regulatory role in the transcription of *Dll1* (Galceran et al., 2004; Hofmann et al., 2004), these studies have been largely restricted to *in vitro* analyses. Therefore, in order to accurately assess the role of Wnt signalling in the regulation of *Dll in vivo* in the PSM, I used an inhibitor-based approach, similar to that of the Notch inhibitor experiments.

The main inhibitor of the Wnt signalling pathways in this chapter is the small molecular inhibitor XAV939 (XAV). XAV has previously been characterised as a potent inhibitor of Wnt3a-stimulated luciferase reporters in HEK293 cells, and SW480 cells (a colorectal cancer line with a truncated APC) (Huang et al., 2009). XAV mode of action is to inhibit the poly-ADP-ribosylating enzymes Tankyrase 1 and 2, which interact with the Axin protein and stimulate its degradation via the ubiquitin-proteosome pathway (Huang et al., 2009). Although the inhibitor has been used on zebrafish at a concentration of 5μM, there are no publications in which the inhibitor has been used in mouse embryos. Because of this, I had to ascertain its effectiveness with a titration assay. I addressed the efficacy of a wide range

of concentrations in an *in vitro* PSM explant assay in order to establish the lowest effective concentration of the drug to be used in subsequent experiments.

Although the use of XAV in cell culture has been shown to be specific to antagonising Wnt signalling, I also used another Wnt inhibitor: Soluble Frizzled Receptor protein 2 (sFRP2), which acts by sequestering Wnt ligands and preventing them from binding to the Frizzled receptor. The purpose of using another Wnt inhibitor which targets a different part of the pathway was to strengthen our argument that the results can be attributed to a loss of Wnt activity.

The aim of this first chapter is to thoroughly investigate the transcriptional regulation of the Delta-like ligands in the PSM of chick and mouse embryos, and to determine whether the transcriptional regulation of these ligands serve as a point of cross-talk between the Notch and Wnt signalling pathways.

Mature mRNA transcripts of Delta-like ligands do not oscillate in the vertebrate PSM

To investigate exactly how the expression of Delta-like ligands is regulated in the vertebrate PSM, I initially used *in situ* hybridisation to look at the localisation of the mature Delta-like transcripts in the PSM of both wholemount mouse embryos at 10.5 days post fertilisation (E10.5) and wholemount chicken embryos at HH10 and HH11. In the mouse, I investigated the expression profile of the ligands *Dll1* and *Dll3*, which are the only DLL ligands to be expressed in the mouse PSM; and *Dll1* in the chick, which is the only DLL ligand to be expressed in the chick PSM.

I found that there was no oscillatory activity of mature mRNA whatsoever in the cases of *cDll1* (n=5/5) (Figure 1.1 B), *mDll1* (n=25/25) (Figure 1.1 C), *mDll3* (n=15/15) (Figure 1.1 D) ligands. During the colour revelation step in the *in situ* hybridisation protocol, for *mDll1* the rostral PSM would be the first region to be coloured, and as the revelation time was lengthened the expression domain expanded further caudally down the PSM until the whole tissue turned black (Figure 1.1 G-I). In the case of *cDll1*, the strongest regions of expression were found in the anterior limit of the PSM and in the caudal PSM (tail bud), but as the revelation time was lengthened the mid-PSM would become stained as well. These findings were in stark contrast to the expression profile of other clock genes (Figure 1.1A), for example *mHes7*, where distinctly different phases can be seen in different mouse embryos irrespective of the incubation time in the colour revelation step (Figure 1.1E, F).

I deduced from these data that the mature mRNAs for the Delta-like ligands in the PSM are not dynamically expressed within this tissue. However, I wanted to further investigate whether the transcription of the *Dll* ligand promoters is dynamically regulated in the PSM by

looking at the newly-transcribed, pre-spliced intronic mRNA, which is less stable than mature mRNA in which the introns have been removed due to splicing.

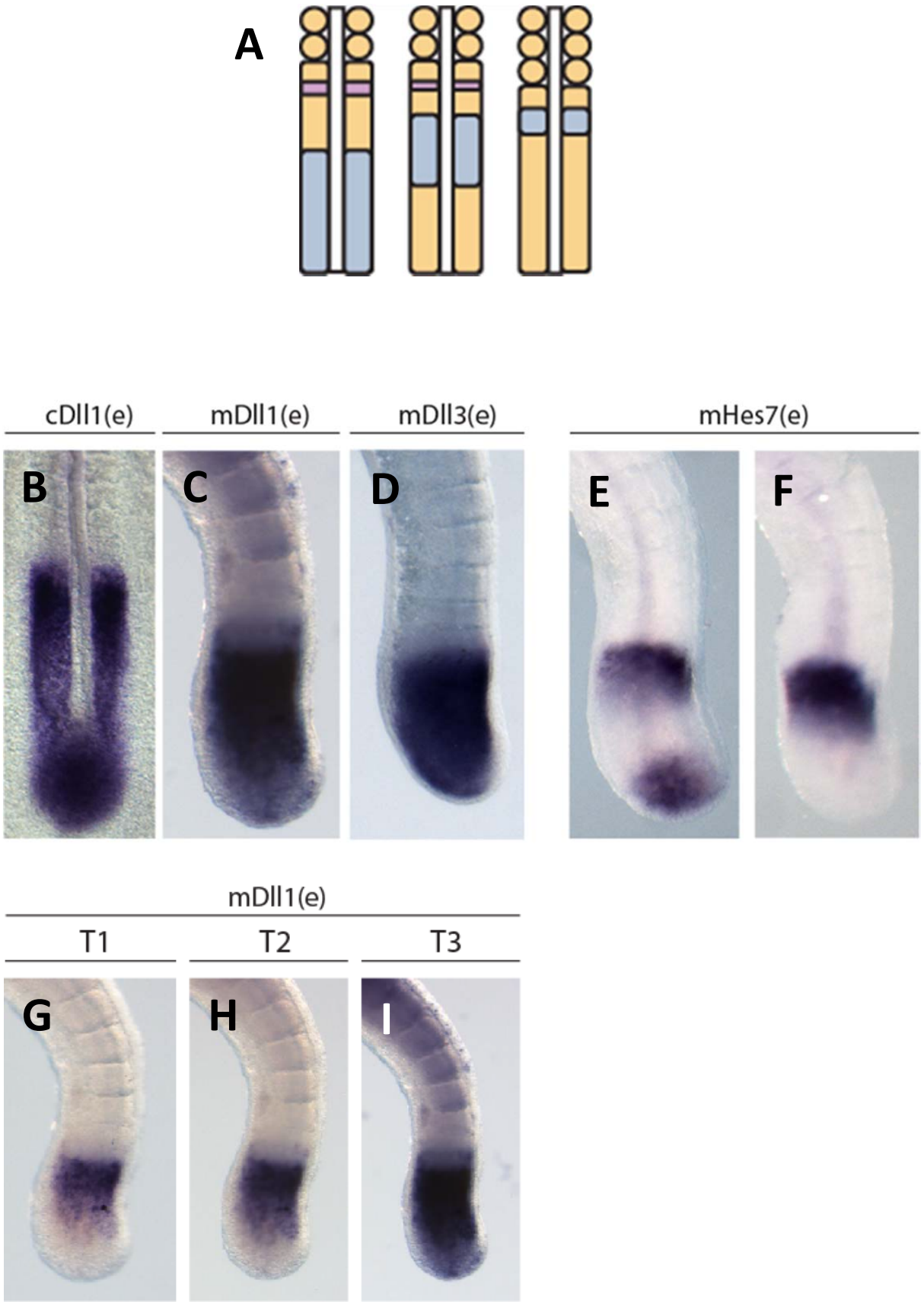
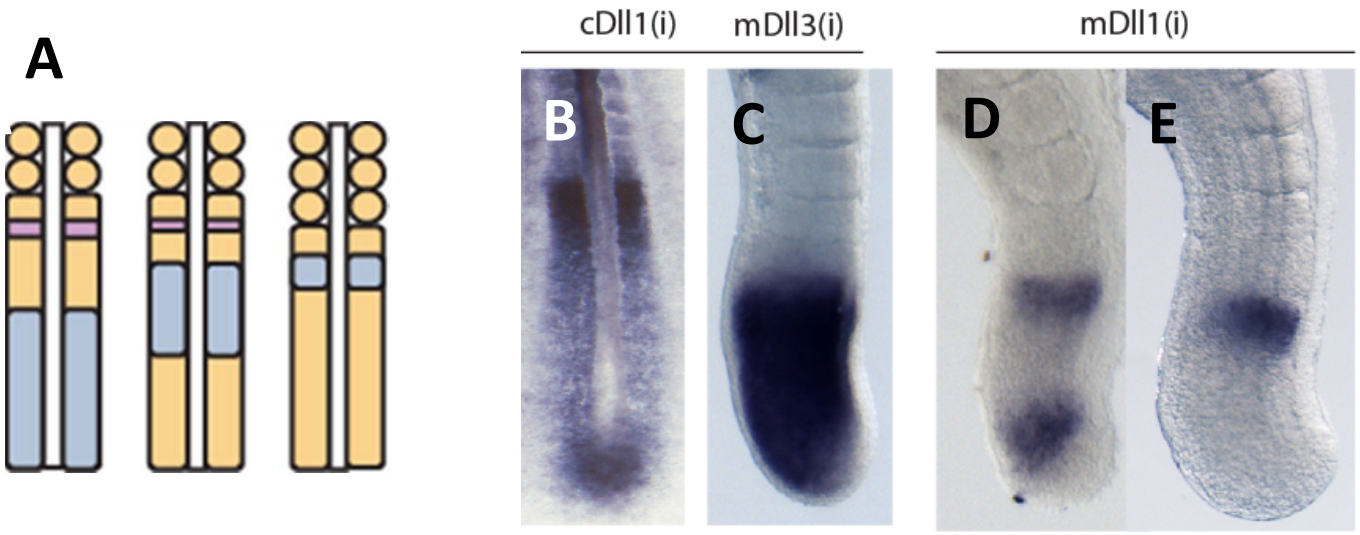

A
cDll1(e)
mDll1(e)
mDll3(e)
mHes7(e)
B
C
D
E
F
mDll1(e)
T1
T2
T3
G
H
I

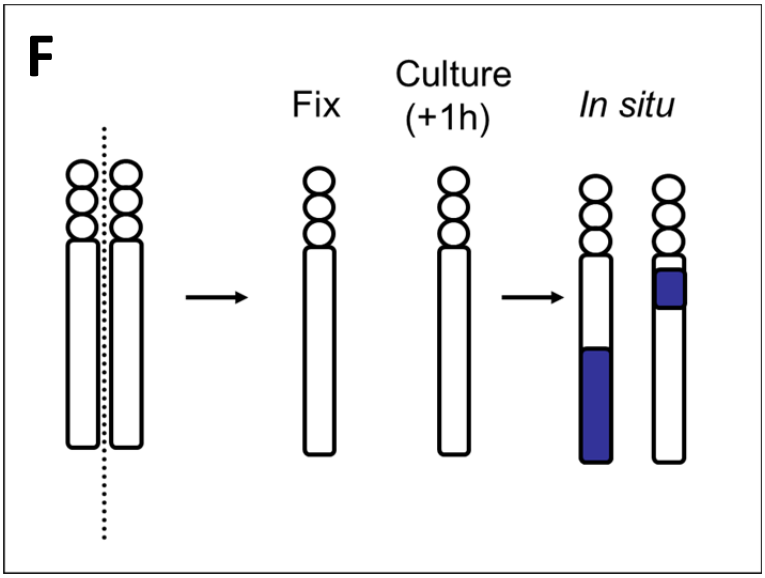
Figure1.1 Mature mRNA transcripts for *Dll1* ligands do not oscillate across mouse or chick PSM

Expression analysis was carried out on the Delta-like ligands that are expressed in the vertebrate PSM in wholemount embryos, to ascertain whether they exhibit dynamic transcription in the PSM in a manner of a clock gene (A). In situ hybridisation of *Dll1* in the chick (B) and mouse (C) PSM, and *Dll3* in the mouse PSM (D) revealed that the exonic transcripts do not oscillate in this tissue, when compared to the known clock gene *Hes7* (E, F). Time course revelation (T1, T2 and T3) during the colouring process for *mDll1* shows that the rostral PSM is the first region of the PSM where mRNA is detected. As the colour development continues the mRNA in the remaining PSM was detected in a rostro-caudal fashion (G-I).

Nascent *Dll1* oscillates across the mouse PSM, whereas *cDll1* and *mDll3* do not.

The next step was to look use a probe that detected nascent pre-spliced "intronic" mRNAs for the *Dll* ligands in the mouse and chicken PSM. Because the intronic mRNA transcripts are normally less abundant than exonic mRNA (see Materials and Methods), I used a less stringent *in situ* hybridisation protocol. In the case of *cDll1* (n=6) (Figure 1.2 B) and *mDll3* (n=10) (Figure 1.2 C), the expression patterns seen in wholemount embryos using an intronic probe were the same as those of the corresponding exonic *in situ* hybridisation results. However, when I looked at *mDll1* expression using an intronic probe, I saw different expression patterns in the PSM of different embryos. The different expression patterns suggested this may reflect an oscillatory expression profile since the patterns very closely resembled those seen for the well-established cyclic genes: Phase1 n=6/28 (Figure 1.2 D), Phase2 n=12/28 (not shown), Phase3 n=10/28 (Figure 1.2 E). Even though I saw this behaviour in a number of wholemount embryos, I wanted to confirm these findings with a technique called 'Fix and Culture', in which the tail of an embryo is bisected down the midline, generating two identical explants: one PSM explant is cultured for 1 hour longer than the other explant (Figure 1.2 F). By comparing *mDll1(i)* expression using an intronic probe in the two PSM explants of the same embryo treated in this way I could determine if the expression was dynamic. Using this technique, I was able to confirm that *mDll1(i)* showed different phases in both explants (Figure 1.2 G) (n=5), similar to that of the clock gene *mHes7* (n=2) (Figure 1.2 H). This confirmed that *mDll1(i)* oscillates in the PSM in the manner of a clock gene, therefore its transcription is likely to be controlled by the segmentation clock.


A
cDll1(i)
mDll3(i)
mDll1(i)
B
C
D
E


F
Fix
Culture
(+1h)
In situ

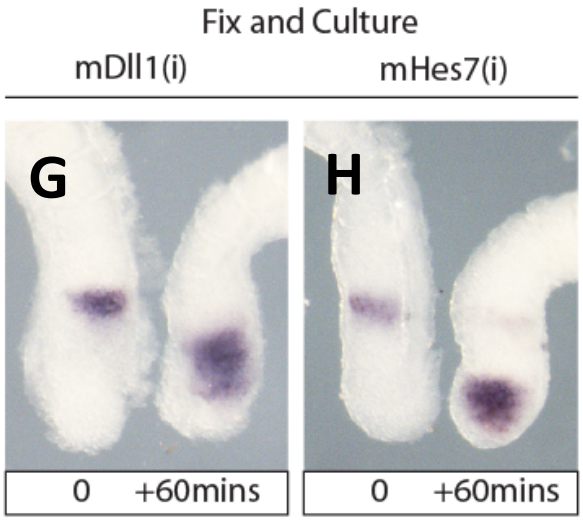

Fix and Culture
mDll1(i)
mHes7(i)
G
H
0
+60mins
0
+60mins

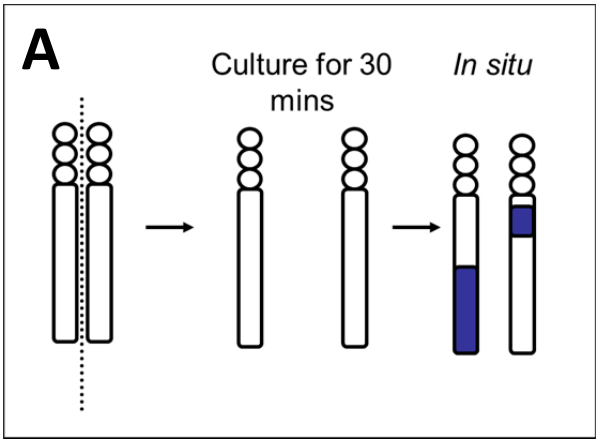
Figure 1.2 Intronic mRNA transcripts for *mDll1* oscillates in mouse PSM

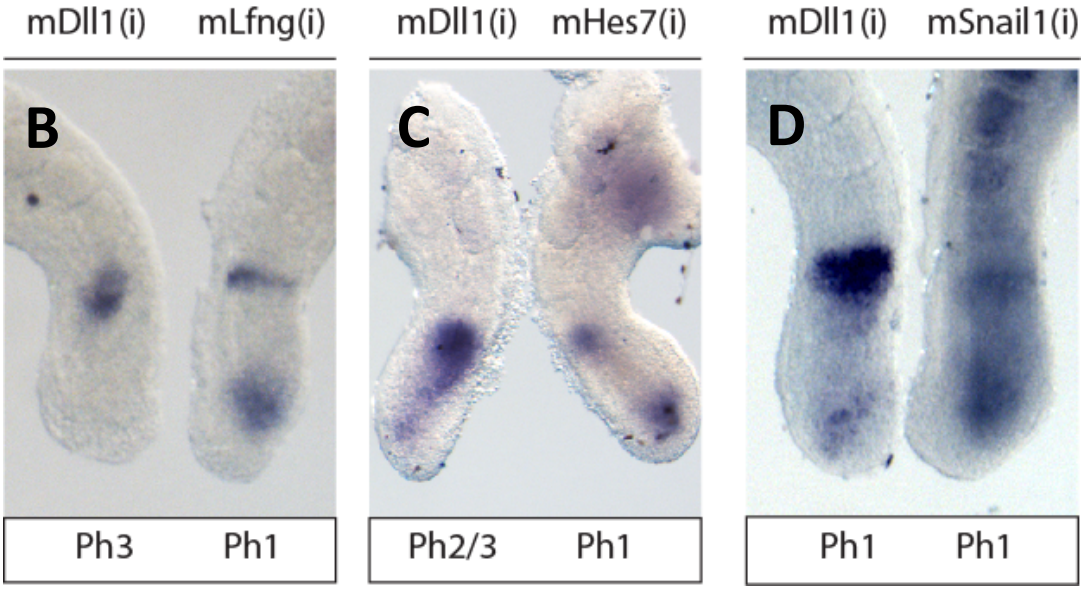
Analysing the pre-spliced mRNA for the Delta-like ligands in wholemount embryos shows that *Dll1* in the chick PSM (B) and *Dll3* in the mouse PSM (C) do not oscillate across the PSM in the manner of a clock gene (A). However, the intronic mRNA for *mDll1* does show different phases in the same profile as a clock gene (D, E). The 'fix and culture' of mouse embryo explants (F), followed by *in situ* hybridisation analysis for *mDll1(i)* revealed that the 'fix' explant (marked '0') was in Phase 3, whereas the 'culture' explant (marked '+60 mins') had entered into Phase 2 in the next cycle (G). This was similar to the fix and culture analysis carried out for *mHes7* (H).

mDll1(i) oscillates in phase with Wnt targets and out of phase with Notch targets

To ascertain which signalling pathway may be primarily responsible for the dynamic expression of *mDll1(i)*, I compared its expression profile with that of other Notch and Wnt cyclic targets. To do this I used a 'Culture-Culture' experiment, where the embryo tail was again bisected down the midline, and this time both explants were fixed after 30 minutes in culture. One explant was then analysed by *in situ* hybridisation for *mDll1(i)* and the other explant for a different cyclic gene (Figure 1.3A). *mDll1(i)* appears to oscillate out of phase with the Notch targets *Lfng* (n=2/8 in phase) (Figure 1.3 B) and Hes7 (n=0/4 in phase) (Figure 1.3 C). The same observation was made using double fluorescent *in situ* hybridisation when comparing the expression profiles of *mDll1(i)* and *mHes7(i)* (n=5) (Figure 1.3 E-G). . The latter analysis revealed that *mDll1(i)* appears to be oscillating out of synchrony with *mHes7(i)*, although the expression domains are not mutually exclusive and there is some overlap in some expression domains (for example when *Dll1(i)* is in Phase 3, and *Hes7* is in a late Phase 2).

In contrast, when I compared *mDll1(i)* expression with that of the Wnt target *Snail1(i)*, the phases appeared to largely overlap, indicating that they appear to oscillate in synchrony (n=3/3 in phase) (Figure 1.3 D). Since cyclic targets of the FGF pathway oscillate in phase with other Notch targets (Dequeant et al. 2006), I did not compare these to *mDll1(i)*. This indicates that the primary signalling pathway regulating *mDll1(i)* in the mouse PSM is Wnt, rather than Notch, because the nascent mRNA transcripts for *mDll1* oscillate more in synchrony with the Wnt target *mSnail1(i)* than for the Notch target genes *mHes7(i)* and *mLfng(i).*


A
Culture for 30 mins
In situ


mDll1(i)
mLfng(i)
B
Ph3
Ph1
mDll1(i)
mHes7(i)
C
Ph2/3
Ph1
mDll1(i)
mSnail1(i)
D
Ph1
Ph1

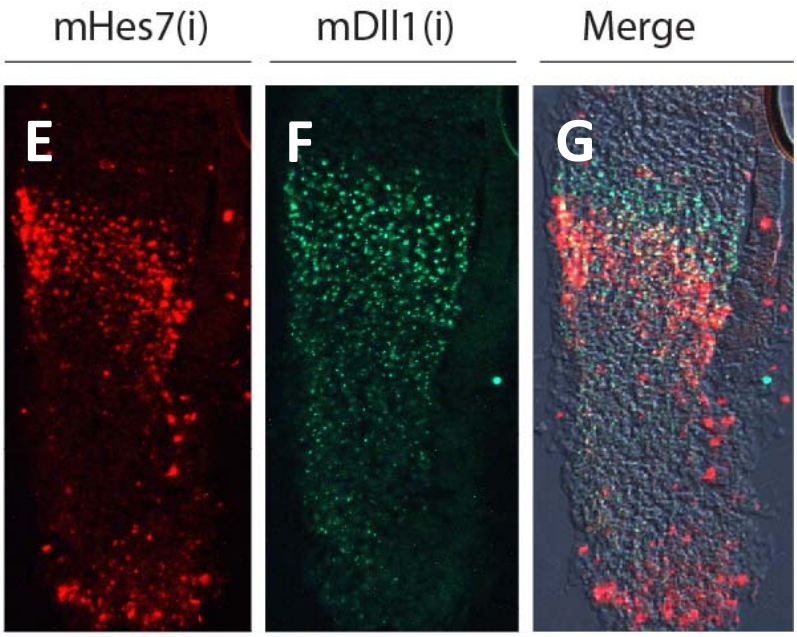

mHes7(i)
mDll1(i)
Merge
E
F
G

Figure 1.3 mDll1(i) oscillates out of phase with Notch targets, but in phase with the Wnt target mSnail1(i)

'Culture-culture' analysis of E10.5 mice embryos: the embryo tail was bisected down the midline, the two explants were then fixed and analysed by *in situ* hybridisation to compare the oscillation pattern of *mDll1(i)* with cyclic genes from the Notch and Wnt signalling pathways (A). When the oscillations of *mDll1(i)* were compared with the oscillations of the Notch pathway targets *mLfng(i)* (B) and *mHes7(i)* (C) they were consistently in different phases. However, when the oscillations of *mDll1(i)* and *mSnail1(i)* were compared (D), the phases were relatively similar, indicating that *mDll1(i)* may be regulated by the Wnt pathway. Comparing the expression profiles of *mDll1(i)* and *mHes7(i)* with double fluorescent *in situ* hybridisation in the same embryo revealed that *mDll1(i)* expression appears to be out of phase with *mHes7(i)* expression (E, F, G).

Inhibiting Notch signalling pharmacologically does not significantly reduce levels of *mDll1(i)* expression, nor does it affect *mDll1(i)* oscillations

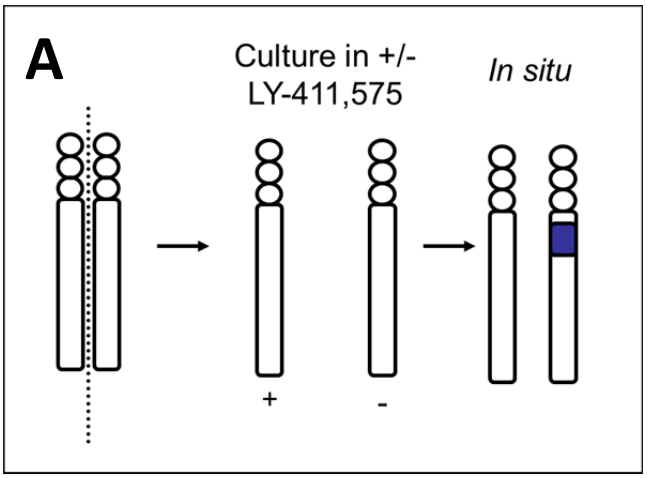
In order to fully understand how the transcription of *mDll1(i)* is regulated in the mouse PSM, I next decided to look at the effects of inhibiting Notch and Wnt signalling pathways. To do this, I used a small molecular inhibitor approach to investigate which pathways regulate dynamic *mDll1(i)* transcription in the mouse PSM. Firstly, to inhibit the Notch signalling pathway I used the inhibitor LY411575 (LY). LY functions in the same way as previously used Notch inhibitors, such as DAPT, by inhibiting the γ-secretase enzyme, which thereby prevents cleavage of the Notch Intracellular domain (NICD) which normally occur following ligand activation, thereby preventing the downstream transcription of Notch pathway targets (Geling et al., 2002).

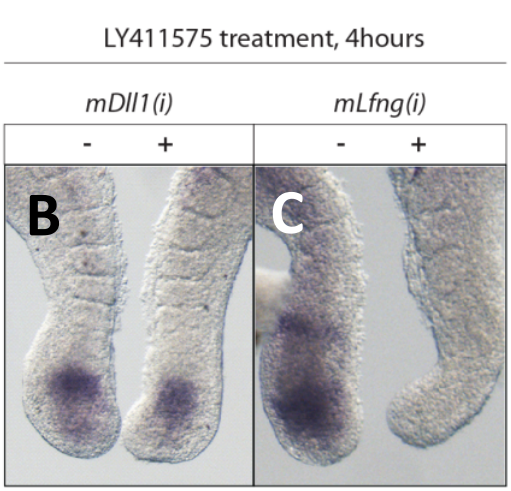
In these experiments the mouse embryo tails were bisected down the midline, generating identical explants with a few (4-6) posterior somites. Then both explants were cultured *in vitro* for the same time period: one explant was incubated in the presence of LY at 10nM, and the other explant in the equivalent volume of DMSO in mouse culture media. After 4 hours of incubation, the explants were fixed, and then *in situ* hybridisation analysis was performed on the explants to detect *mDll1(i)* transcription levels (Figure 1.4 A). The level of *mDll1(i)* transcription does not appear to be affected after 4 hours of Notch inhibition (n=6/6) (Figure 1.4 B), as opposed to the control Notch target gene, *mLfng(i)*, which is completely abolished in the LY-treated explants (n=5/5) (Figure 1.4 A).

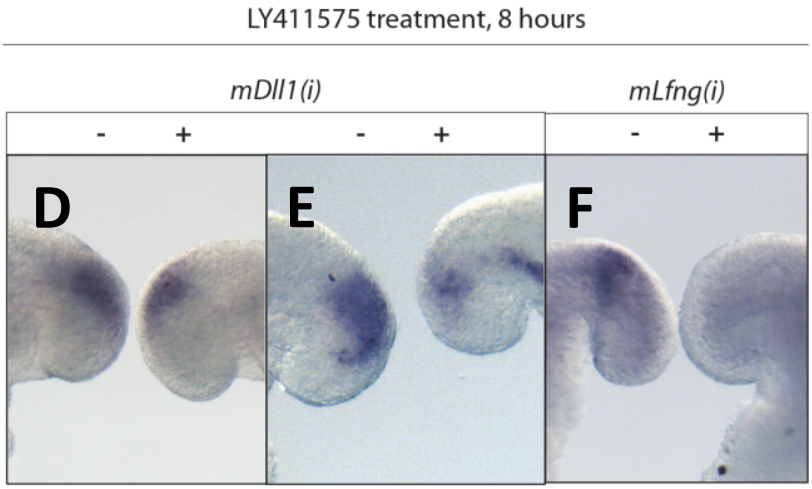
Since there were no observed effects of inhibiting Notch signalling on the level of *mDll1(i)* expression, I decided that 4 hours was possibly too short a time period to observe any potential down regulation, and therefore decided to increase the incubation period with LY

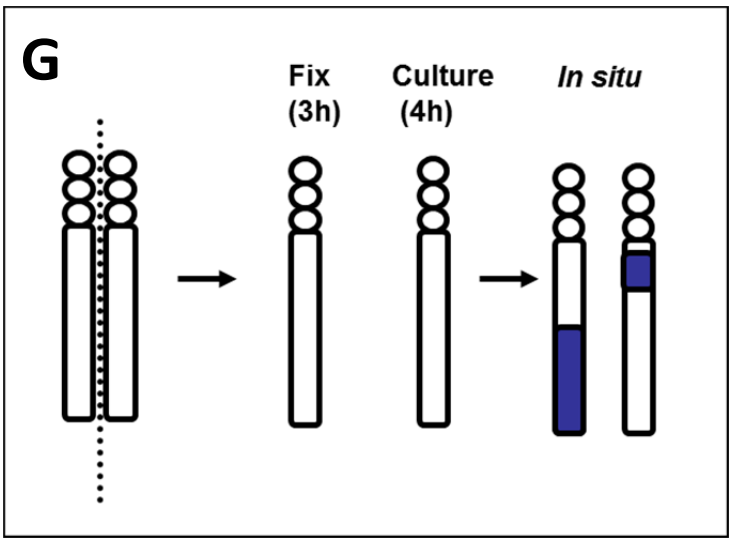
in order to deduce whether Notch signalling has a more indirect role in the regulation of *mDll1(i)* transcription. I therefore increased the incubation time of the explants to 8 hours, and while the level of *mDll1(i)* expression was unchanged in roughly half the samples (n=7/13) (Figure 1.4 D), the other half of the samples showed some down regulation in the level of *mDll1(i)* (n=6/13) (Figure 1.4 E), indicating that the Notch pathway does have some, albeit indirect, regulatory role in the expression of *mDll1(i).* However, the level of down regulation in these samples was never as strong as for the control, *mLfng(i)*, which showed consistent abolition of expression (n=7/7) (Figure 1.4 F).

To investigate whether the primary role of Notch signalling in the segmentation clock is to synchronise the oscillations of *mDll1(i)* rather than to initiate them, as has been reported to be the case in zebrafish (Jiang et al.2000), I next used LY to perform a 'fix and culture' assay, where one explant was incubated in the presence of the drug for 3 hours, and the other explant for 4 hours, before fixing and analysing by *in situ* hybridisation (Figure 1.4 G). If Notch signalling is affecting the oscillations of *mDll1(i)*, then it should be expected that since both explants are treated, albeit for different time periods, they should both have stopped oscillating and should therefore display similar phases. However, different phases were observed for *mDll1(i)* in the explants (n=5/7 showing different phases) (Figure 1.4 H), similar to the different phases observed for the Notch cyclic gene *mLfng(i)* (n=2/2 showing different phases, not shown) indicating that Notch inhibition does not affect the oscillations of *mDll1(i)*.


A
Culture in +/-
LY-411,575
In situ
+
-


LY411575 treatment, 4hours
mDll1(i)
mLfng(i)
- + - +
B
C


LY411575 treatment, 8 hours
mDll1(i)
mLfng(i)
- + - + - +
D
E
F


G
Fix
(3h)
Culture
(4h)
In situ

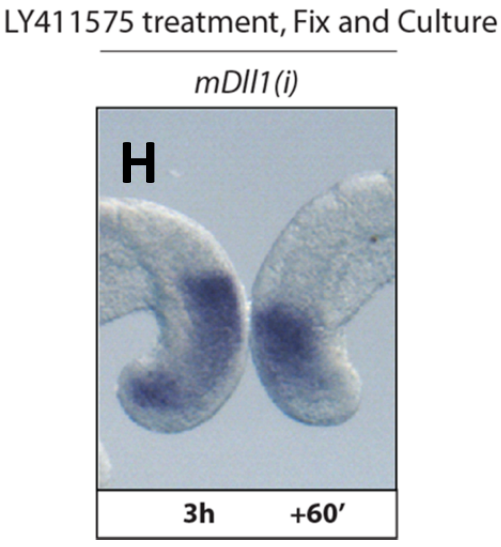

LY411575 treatment, Fix and Culture
mDll1(i)
H
3h
+60'

Figure 1.4 Inhibition of Notch signalling does not significantly affect the expression levels or oscillations of *mDll1(i)*

Mouse embryo explants were dissected and cultured either in the presence of LY, before being fixed and analysed by *in situ* hybridisation (A). After 4 hours treatment, there was no significant difference in the level of *mDll1(i)* expression (B), compared to the Notch target *mLfng(i)* (C), for which the expression is completely abolished after 4 hours. When the period of incubation with the drug is increased to 8 hours, in half the samples there is no down regulation in the drug-treated explant (D), but for the other samples there is a small level of down regulation in the drug-treated explant (E), while the expression for *mLfng(i)* is completely abolished (F). The ability of *mDll1(i)* to cycle in the absence of Notch signalling was ascertained by both explants were treated with LY for either 3 hours ('fix') or 4 hours ('culture'), before fixing and analysis with *in situ* hybridisation (G). This revealed that *mDll1(i)* oscillations continue in the absence of Notch signalling (H).

XAV-939 effectively down regulates Wnt targets at 100µM, but only partially down regulates *mDll1(i)*

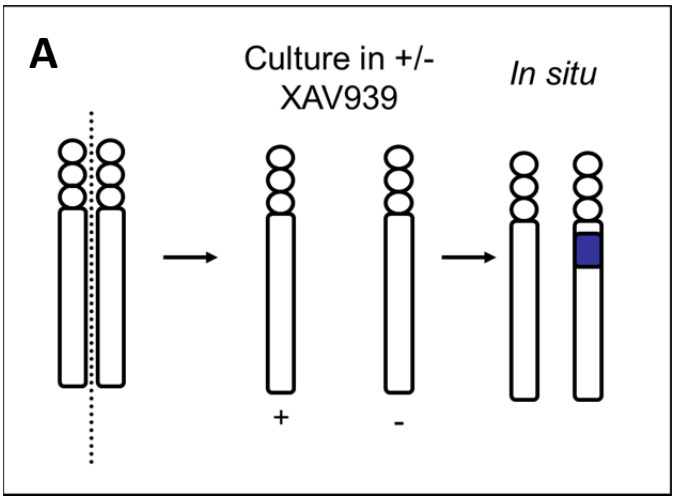
To address whether Wnt signalling regulates the transcription of *mDll1(i)*, I used the small molecular inhibitor XAV939 (XAV). XAV acts to stabilises Axin2 in the β-catenin destruction complex, thereby preventing the transcription of downstream Wnt targets. However, to date the drug has only been used in cell lines zebrafish embryos at a concentration of 5µM (Huang et al. 2009).

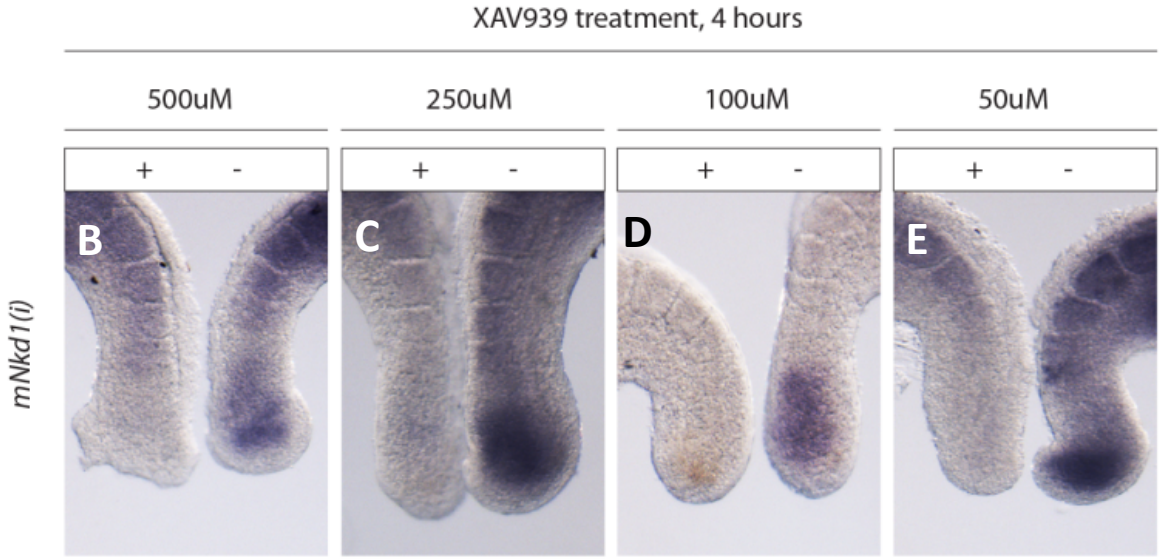
In order to assess the effectiveness of XAV of inhibiting the expression of Wnt target genes in mouse embryo tails, I performed a titration experiment to determine the appropriate concentration to use in subsequent experiments. The range of concentrations tested was 500µM, 250µM, 100µM and 50µM (n=3 for all concentrations) (Figure 1.5 B-E), and the effectiveness of the drug was determined by analysing the level of inhibition of the Wnt target *mNkd1(i)* in treated explants after 4 hours. It was shown that the optimum concentration to use was 100µM (n=3/3 show total down regulation) (Figure 1.5 D), as this was the lowest concentration at which no expression of *mNkd1(i)* was observed in the treated explants.

Having established the optimum concentration of XAV, we then used the inhibitor in a 'drug culture' assay, similar to the Notch inhibition culture experiment, whereby E10.5 mouse embryo tails were bisected down the midline and cultured for 4 hours: one explant in the presence of the drug at 100µM in culture media, and the other explant in the equivalent volume of DMSO in culture media; before fixing the tissue and performing *in situ* hybridisation analysis to determine the level of *mDll1(i)* down regulation in the absence of Wnt signalling (Figure 1.5 A).

In mouse explants treated with 100 µM XAV, we observed some down regulation of *mDll1(i)* in roughly half the samples (n=5/9) (Figure 1.5 G), but in the other samples there appeared to be no difference in levels of *mDll1(i)* expression (n=4/9) (Figure 1.5 F). None of the samples showed complete down regulation as for the control Wnt target gene *mAxin2(e)* (n=3/3) (Figure 1.5 H).

Having observed a mild down regulation of *mDll1(i)* following inhibition of Wnt signalling after 4 hours, we extended the period, to see if this caused more effective down regulation. When the incubation period was extended to 8 hours we observed that in all cases *mDll1(i)* transcription was down regulated (n=8/8) (Figure 1.5 I), but never completely abolished, in contrast to *mNkd1(i)* (n=3/3) (Figure 1.5 J). This was surprising given published reports that *mDll1(i)* expression is reliant upon Wnt signalling *in vitro* (Hofmann et al., 2004). Our *in vivo* experiments show that even after Wnt signalling is inhibited for 8 hours there is still a low level of *mDll1(i)* transcription which appears to be Wnt-independent.


A
Culture in +/-
XAV939
In situ
+
-


XAV939 treatment, 4 hours
500uM
250uM
100uM
50uM
+ -
+ -
+ -
+ -
mNkd1(i)
B
C
D
E

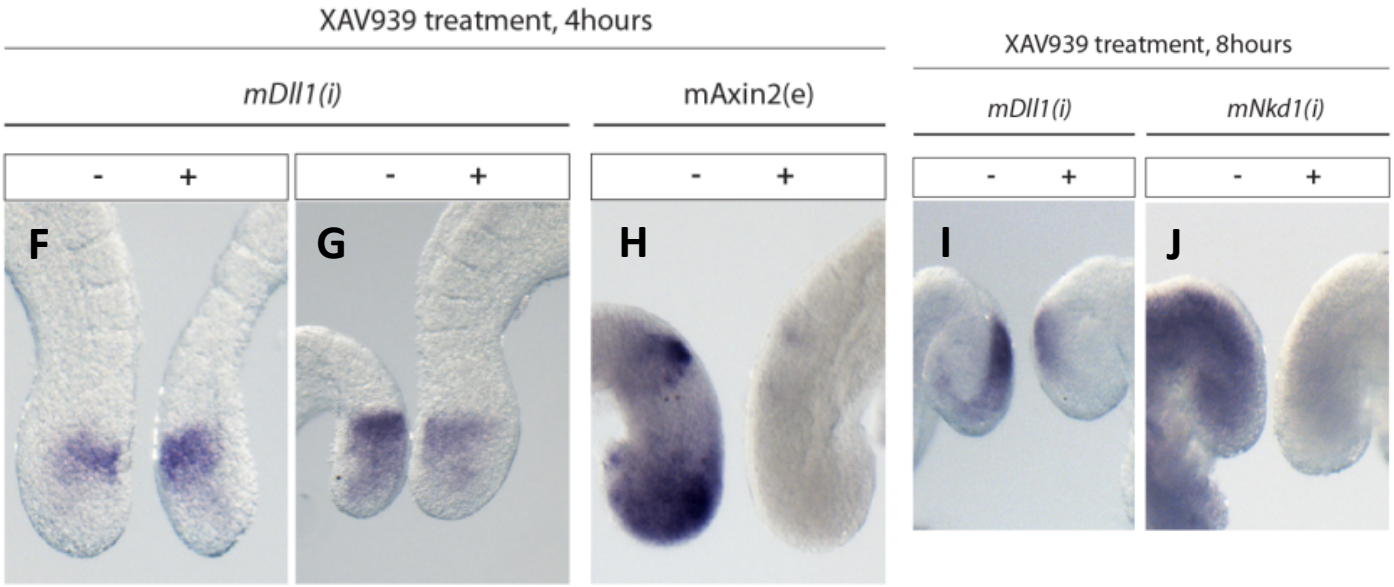

XAV939 treatment, 4hours
mDll1(i)
mAxin2(e)
XAV939 treatment, 8hours
mDll1(i)
mNkd1(i)
- +
- +
- +
- +
- +
F
G
H
I
J

Figure 1.5 XAV939 is an effective inhibitor of Wnt signalling in the mouse PSM, and partially down regulates *mDll1(i)* expression

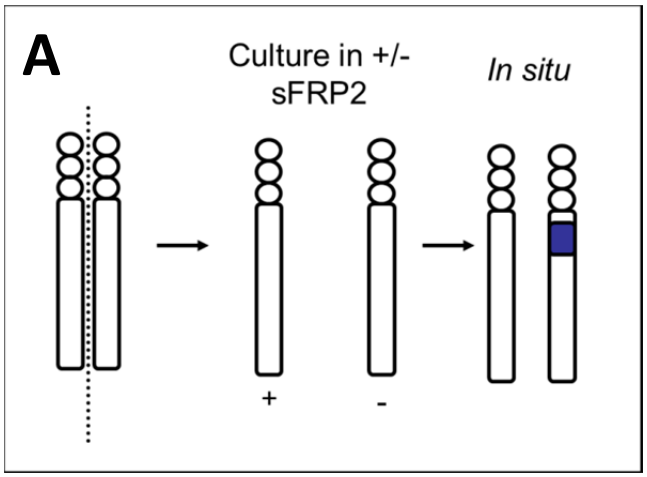
To determine the effective concentration of XAV939 in the mouse embryo, a titration experiment was performed (A), in which various concentrations of the drug (500µM (B), 250µM (C), 100µM (D), and 50µM (E)) were tested on mouse embryo explants: one explant was incubated with the drug, the other in control media, for 4 hours, before fixing and *in situ* hybridisation analysis for the Wnt target *mNkd1(i)*. The lowest concentration of the drug that consistently abolished expression of the drug was 100µM. This concentration was then used in the remainder of the experiments with XAV939.

Mouse embryo explants dissected and cultured in the presence/absence of XAV, before being fixed and analysed by in situ hybridisation. After 4 hours treatment, in half the samples analysed there was no difference in the level of *mDll1(i)* expression (F), however there was a significant difference in the level of *mDll1(i)* expression in roughly half the samples analysed (G). *mAxin2(e)*expression is completely abolished after 4 hours (H). Following 8 hour incubation, *mDll1(i)* is down regulated in all drug-treated explants (I), but is not completely abolished. In contrast under these conditions the Wnt target *mNkd1(i)* is completely abolished (J).

The Wnt inhibitor Soluble Frizzled Protein Receptor phenocopies the effects of XAV in *mDll1(i)* down regulation

To support and verify the data with XAV, I repeated the assays using a second Wnt inhibitor, the Soluble Frizzled Receptor Protein 2 (sFRP2), which is a competitive inhibitor of the Frizzled receptor that acts by sequestering Wnt ligand, thereby preventing receptor interaction with ligand and downstream transcription of Wnt targets.

Because this inhibitor is a protein, it was required to be used at a relatively high concentration because its cell permeability is not as efficient as for small molecular inhibitors such as XAV. Since previous unpublished work has shown that sFRP2 down regulates targets of Wnt signalling at a concentration of 10 µg/ml, I used this concentration in the drug culture assay to determine its effectiveness at down regulating *mDll1(i)* (Figure 1.6 A). At this concentration, the Wnt target gene *mAxin2(e)* was effectively down regulated (n=4/4) (Figure 1.6 C), but the level of *mDll1(i)* RNA was unaffected (n=6/6) (Figure 1.6 B). However, when the concentration was increased to 20µg/ml, again we found that the Wnt target Axin2 was down regulated (n=2/3) (Figure 1.6 F), and that while in half the samples there was no difference in transcription levels of *mDll1(i)* (n=2/4) (Figure 1.6 E), we found that in half the samples there was some down regulation of *mDll1(i)* (n=2/4) (Figure 1.6 F). These results phenocopy what was observed for XAV-treated explants at a concentration of 100µM, and strengthens our argument that the effects of *mDll1(i)* down regulation are caused by abolished Wnt activity, instead of other secondary effects of using inhibitors.


A
Culture in +/-
sFRP2
In situ
+
-

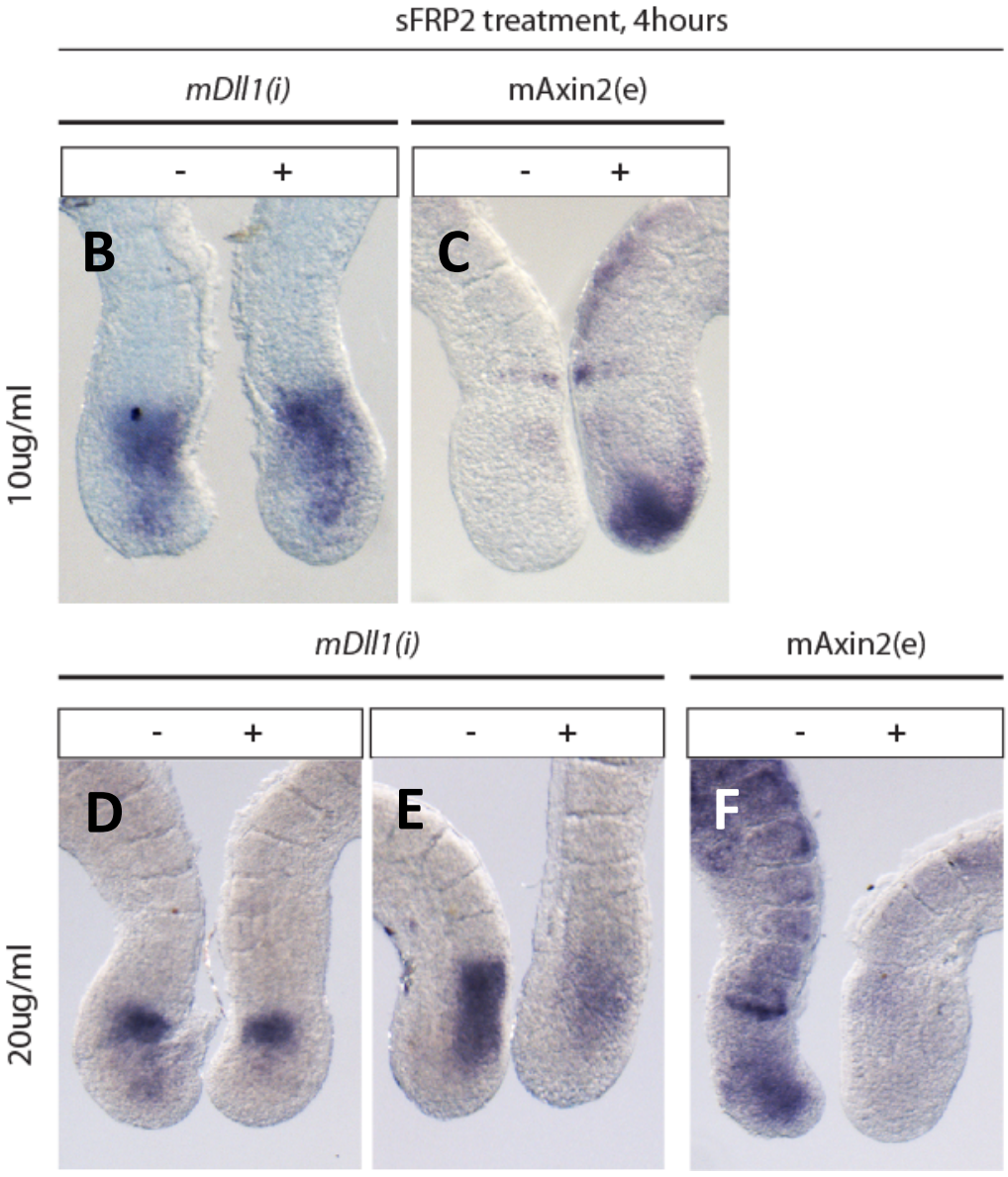

sFRP2 treatment, 4hours
mDll1(i)
mAxin2(e)
- +
- +
B
C
10ug/ml
mDll1(i)
mAxin2(e)
- +
- +
- +
D
E
F
20ug/ml

Figure 1.6 sFRP2 phenocopies the effects of XAV939 on *mDll1(i)* transcription

Mouse embryo tail explants were dissected at E10.5 and cultured in the presence or absence of sFRP2, for 4 hours, before fixing and analysis by in situ hybridisation (A). At 10µM SFRP2, *mDll1(i)* was unaffected (B), compared to *Axin2(e)* (C). With 20µM SFRP2, the level of *mDll1(i)* transcripts was unaffected in 2/4 samples analysed (D), whereas they were down regulated in the other samples (E), but not as strongly as for *mAxin2(e)* (F).

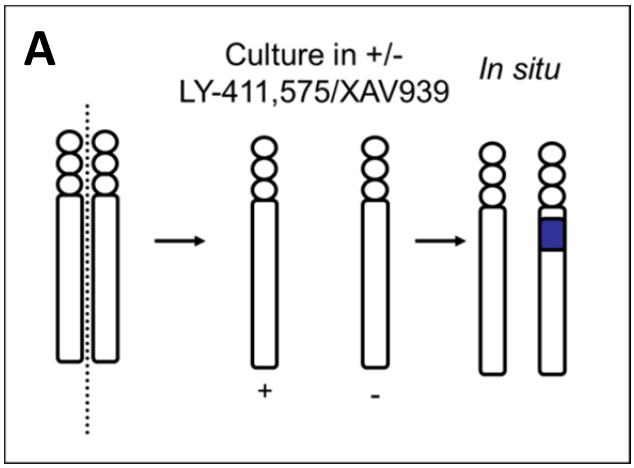
Inhibition of both Notch and Wnt signalling pathways causes the abolition of *mDll1(i)* expression

Since inhibition of either Wnt or Notch signalling did not completely abolish *mDll1(i)* expression, I inhibited both pathways simultaneously to see whether there was any co-dependence for the transcription of *mDll1(i)* (Figure 1.7 A). Strikingly, I found that *mDll1(i)* expression was abolished in the treated explants (n=10/13) (Figure 1.7 B), as were the control Notch target *mLfng(i)* (n=4/4) (Figure 1.7 C) and Wnt target *mNkd1(i)* (n=4/4) (Figure 1.7 D). This data suggests that Notch signalling does have an indirect influence on *mDll1(i)* transcription, and possibly serves to partially compensate for loss of Wnt by maintaining low level *mDll1(i)* transcription in the assay in which explants are cultured in the presence of XAV.

It has previously been reported that the mDll1 promoter contains a number of TBX6-binding sites, and that TBX6 is required for the expression of *Dll1* in cell culture luciferase assays (Hofmann et al., 2004). Moreover, Tbx6 expression is regulated by both Notch and Wnt pathways (Galceran et al., 1999; White et al., 2005). In light of this evidence, I next addressed whether the loss of *mDll1(i)* transcription, following exposure to both Notch and Wnt inhibitors, was due to a loss of *mTbx6(i)* transcription. To do this, we performed a drug culture analysis in which the explants were cultured either in the presence of both LY and XAV939 at 150nM and 100µM respectively, or in control media, for 4 hours. The results showed *mTbx6(i)* was also completely abolished in the absence of Notch and Wnt signalling (n=4/6) (Figure 1.7 F). To determine whether a lack of TBX6 protein was the cause of *mDll1(i)* abolition, I then performed an immunohistochemistry staining for TBX6 protein after 4 hours of exposure to both inhibitors. The level of TBX6 protein was unchanged in the

treated explant compared to the control explant (n=3/3) (Figure 1.7 G), showing that the presence TBX6 protein alone is insufficient for *mDll1(i)* expression, which concurs with previous data (Hoffman et al. 2004). This remains to be confirmed quantitively with the Western blot.

Addition of NucView 488 Caspase -3 substrate (which produces a fluorescent signal for cells in apoptosis) at the end of the drug treatment demonstrated that the explants treated with the two drugs did not show more apoptosis than explants cultured in control media (n=6) (Figure 1.7 E). This shows that the abolition of *mDll1(i)* was not due to the cells dying, but rather because of the effects of down regulated Notch and Wnt signalling in the PSM.


A
Culture in +/-
LY-411,575/XAV939
In situ
+
-

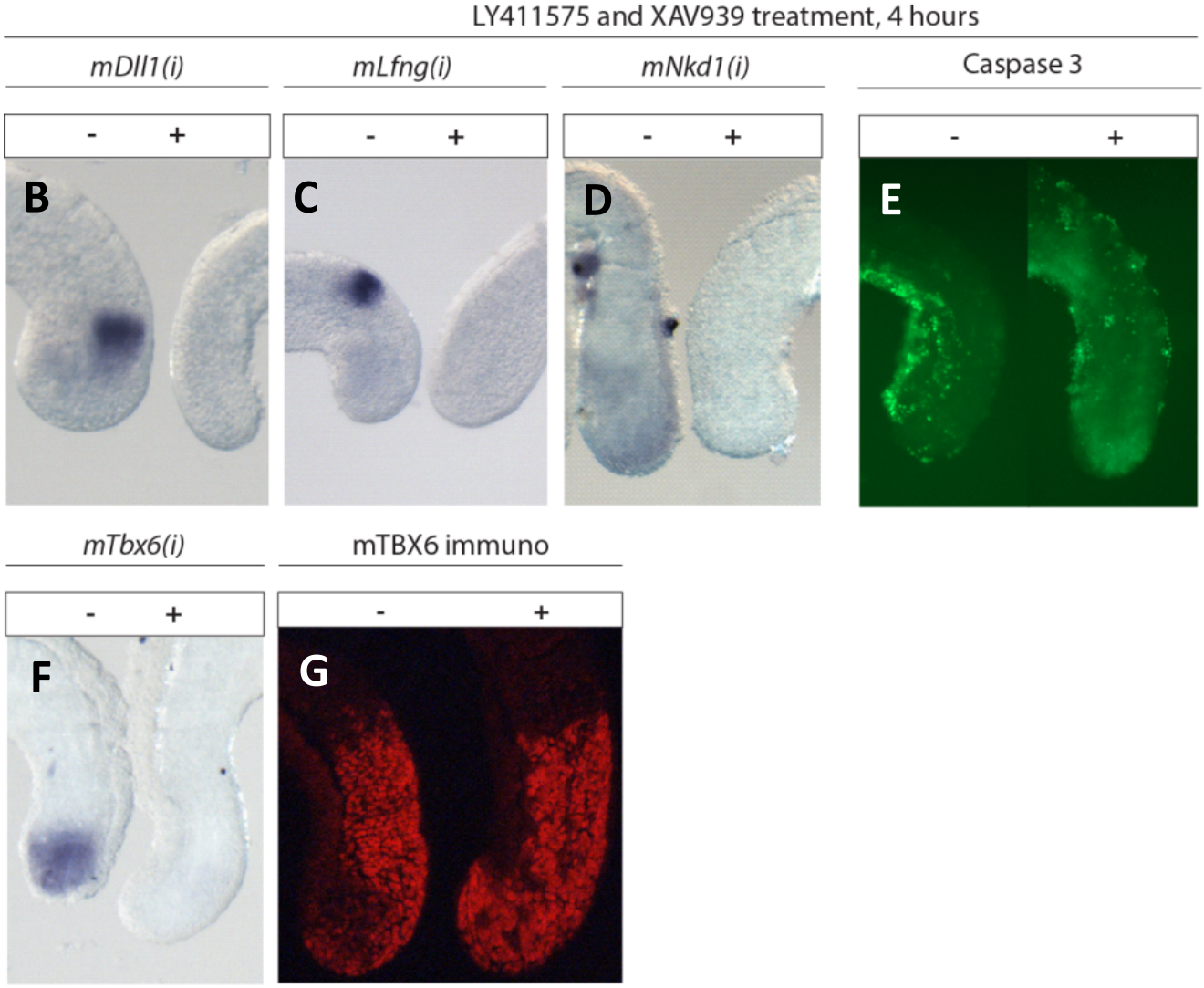

LY411575 and XAV939 treatment, 4 hours
mDll1(i)
mLfng(i)
mNkd1(i)
Caspase 3
- +
B
C
D
E
mTbx6(i)
mTBX6 immuno
F
G

Figure 1.7 Inhibiting Notch and Wnt signalling pathways abolished *mDll1(i)* expression

Mouse explants were treated either in the presence or absence of both LY and XAV inhibitors for 4 hours, followed by fixing and analysis by *in situ* hybridisation (A). In almost all cases, the expression of *mDll1(i)* was completely abolished (B), as were the Notch control *mLfng(i)* (C) and Wnt control *mNkd1(i)* (D),. Levels of caspase-3, which detects levels of apoptosis within cells, were similar in both treated and control explants (E). *mTbx6(i)* was also abolished following this treatment (F), However, immunohistochemistry for TBX6 following Notch and Wnt inhibition revealed no difference in the level of TBX6 protein after 4 hours of LY and XAV treatment (G).

Chapter 2: Are Oscillations required for Somitogenesis?

Introduction

Since the presence of oscillations of gene expression in the PSM is thought to be linked with the process of somitogenesis, we wanted to ask whether the abolition of these oscillations in the PSM of mice and chick embryos would result in the loss of segmentation. It has been shown that in mice which lack Hes7, and therefore have Notch activity across the PSM which is non-dynamic, the somites are abnormally formed. However, the cyclic target genes of the Wnt and FGF pathway are still oscillating in the PSM of these mutant mice, which could explain the fact that segmentation is still occurring (Ferjentsik et al., 2009). This raises the possibility that the presence of oscillations in the PSM is sufficient to ensure that somitogenesis can occur, irrespective of which pathway targets are cycling. Therefore we decided to test this hypothesis by setting out to cease the oscillations of cyclic genes in the chick and mouse model and subsequently determine if somite were able to form under these conditions.

Because the mouse PSM has cyclic gene targets from three signalling pathways: Notch, Wnt and FGF; we needed to establish a system where the oscillations of the cyclic genes from all these pathways could be halted. This would then allow us to observe whether somites are able to form in the absence of these oscillations. In order to do this, we initially investigated the potential of two separate mouse lines: Hes7-/-, which has previously been used to demonstrate that segments continue to form in the presence of non-cyclic Notch activity (Ferjentsik et al., 2009); and a constitutively active Lef1 mouse line, which has stable

amounts of the Wnt transcription factor Lef1 in all the cells of the PSM. The catC-Lef1 mouse was first described to rescue the loss of Wnt3a in the vestigial tail mutant by restoring the expression of Brachyury in the tail bud when analysed by *in situ* hybridisation (Galceran et al., 2001). It was also shown in this paper that although Wnt signalling is constitutively active in the PSM, the level of signalling is not up regulated. In other words, it provides a similar model to the Hes7-/- mouse, i.e. Wnt signalling is constant and not dynamic across the PSM, which would allow us to ask the question: How are Wnt cyclic targets affected by constant levels of Wnt activity in the PSM? Similarly, how are Notch and FGF cyclic targets affected by constant levels of Wnt activity in the PSM? In the event that we are able to demonstrate that Hes7-/- mice have non oscillating Notch and catC-Lef1 mice have non oscillating Wnt, the ultimate aim of this study would be to then cross these two mice lines to establish a line where neither pathway oscillates and then analyse somite formation.

We also performed several studies in the chick to observe the effects on somitogenesis of removing cyclic gene expression. The chick model was useful because only the Notch pathway has cyclic gene targets across the PSM that have been identified by *in situ* hybridisation, which makes it less problematic to halt oscillations entirely. In order to undertake these studies we used an *in ovo* approach. We first set out to establish the effects of long term Notch pathway inhibition on the development of somites *in ovo* using the γ-secretase enzyme inhibitor, LY-411575 (see results Chapter 1 for details). In a complementary approach, we performed *in ovo* electroporation of a plasmid which expresses NICD, in order to assess how the presence of constant Notch activity in the PSM affected the formation of somites.

The expression of *mDll1(i)* is up regulated in the caudal PSM of Hes7-/- mouse embryos

I first wanted to observe the expression profile of *mDll1(i)* (which required the input of both Notch and Wnt signalling to be expressed) in Hes7-/- mice, in order to deduce the effect of constant Notch activity on its transcription. The expression of *mDll1(i)* was much stronger in Hes7-/- embryos than in wild type embryos (Figure 2.1 A, B), and was most strongly up regulated in the caudal PSM in all Hes7-/- embryos tested, which resembled the 'Phase 1' of expression (n=7/7). Up regulation was also seen in the rostral PSM domain and the first 4 to 5 somites (Figure 2.1 B). The up regulation of *mDll1(i)* in the caudal PSM domain of these embryos strongly suggests that Hes7 may be negatively regulating *mDll1(i)* expression in the this region.

Figure 2.1 *mDll1(i)* is upregulated in the Hes7-/- mice, particularly in the caudal PSM

Lateral view of E10.5 mouse tails analysed by in situ In the wild type embryo the expression of *mDll1(i)* is restricted to the PSM (primarily the rostral domain) (A), in the Hes7-/- embryos there is a general up regulation of *mDll1(i)* expression in the E10.5 mouse tail (B), especially in the tail bud. There is also an up regulation of *mDll1(i)* in the first few somites, which appears ubiquitous.

In the catC-Lef1 mouse, Wnt target *mSnail1* does not cycle, whereas the Notch target *mLfng* does

To look at the effects of constant Wnt activity on the oscillation profiles of Notch and Wnt pathway targets, I first performed *in situ* hybridisation for the Wnt cyclic target *mSnail1(e)* in the catC-Lef1 embryos (Figure 2.2). The first observation made was that the level of mRNA transcript is increased dramatically in the catC-Lef1 embryo PSM (after one hour in colour revelation at 37°C they are fully developed, whereas the wild type embryos require five or six hours to become fully developed in these conditions (Figure 2.2 B-F)); this indicates that periodic Wnt activity is required for maintaining the mRNA transcripts of Wnt target cyclic genes at a normal level. The second observation we made was that whilst in the wild type embryos we can see clear patterns of oscillatory expression of *mSnail1(e)* (n=4: one embryo in Phase1 (Figure 2.2 C); one embryo in Phase 2 (Figure 2.2 B); two embryos in Phase3 (not shown), in the catC-Lef1 mutant embryos the expression patterns appeared to be very similar: a rostral and caudal band were always present, which corresponds to Phase 1 of the segmentation clock, with some slight variation in the mid-PSM region (n=6/6). This indicates that dynamic Wnt signalling is required for normal oscillations of Wnt targets such as *mSnail1(e)*, which fits our model.

However, since the catC-Lef1 mice are still capable of producing somites, we predict that there would still be some oscillations of other cyclic genes in the PSM, such as Notch signalling pathway targets. To test this possibility, we analysed the expression profile of the Notch target *mLfng(e)* in these embryos, and found that *mLfng* still produces very clear and distinct phases (Figure 2.2 G-I) (n=3, n=1 for each phase) that are found in wild type embryos, and shows that constant non-dynamic Wnt activity does not affect the oscillations

of Notch target genes. Due to time constraints we were unable to proceed to the next step: the ultimate aim of this study namely to then cross these two mice lines to establish a mouse line where neither pathway oscillates and then analyse somite formation.

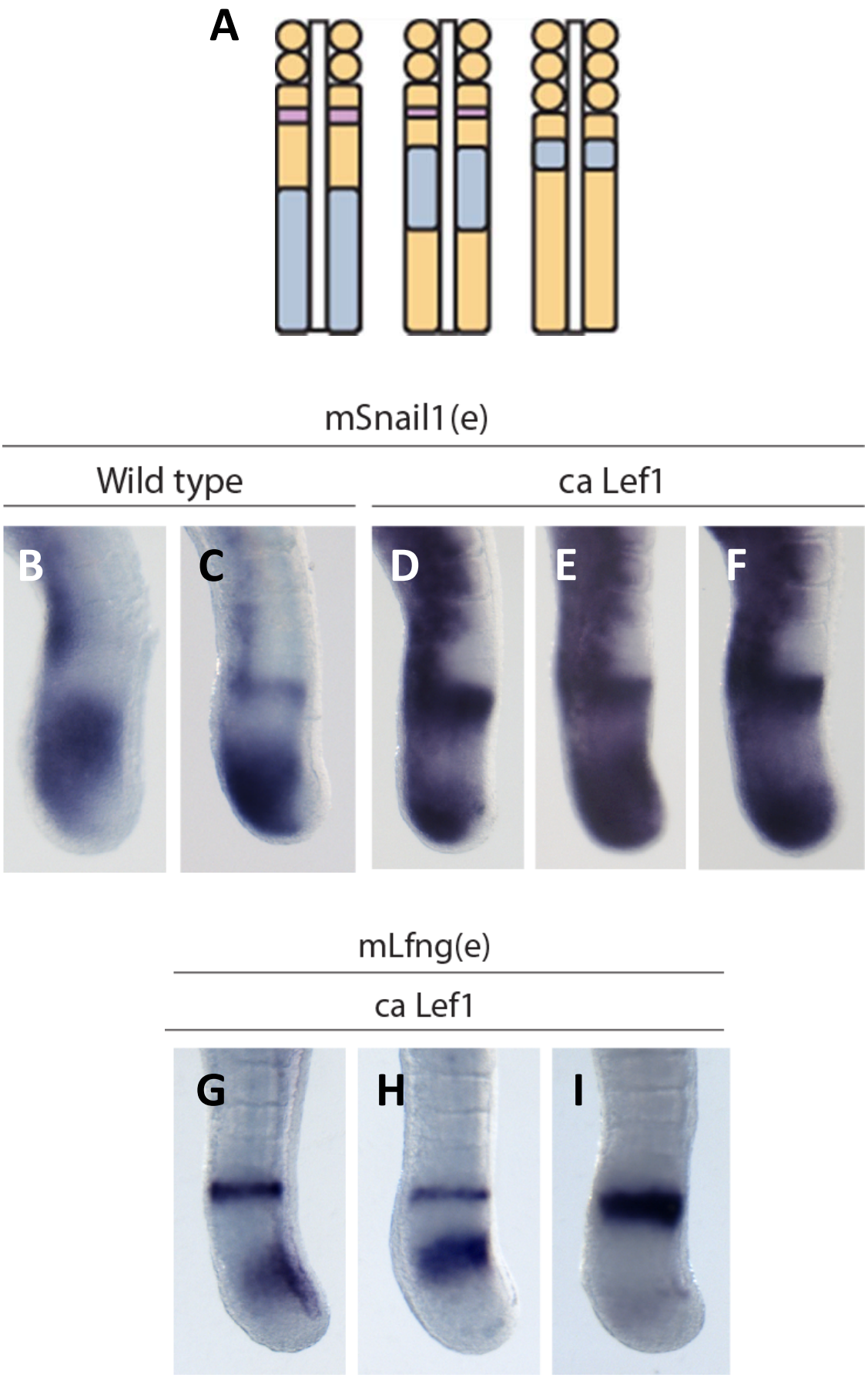

A
mSnail1(e)
Wild type
ca Lef1
B
C
D
E
F
mLfng(e)
ca Lef1
G
H
I

Figure 2.2 Wnt target oscillations are affected in catC-Lef1 mice, whereas Notch target oscillations are not

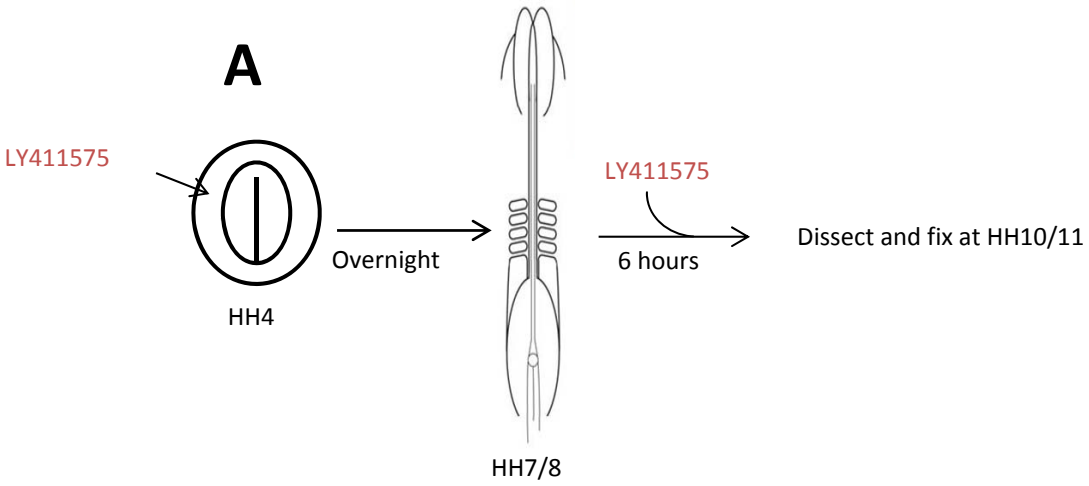
In situ hybridisation analysis of *mSnail1(e)* showed that in E10.5 wild type embryos there are clear and distinct patterns of oscillations (B, C) in the manner of a clock gene (A), whereas in catC-Lef1 embryos the oscillations appear to be exclusively in Phase 1, showing strong expression in both rostral and caudal regions of the PSM, with minor fluctuations of expression levels in the mid-PSM (D - F). These results also reveal an up regulation in the level of mRNA transcript in the PSM of the catC-Lef1 embryos, specifically in the rostral and caudal regions, compared to the wild type embryos. Dynamic expression of *mLfng(e)* in the catC-Lef1 embryos, shows that Notch target gene oscillations continue in the absence of dynamic Wnt activity (G - I).

LY-411575 *in ovo* addition to chicken embryos disrupts somitogenesis

Because there are multiple signalling pathways in the mouse PSM, it is very difficult to establish a system whereby all dynamic expression in the PSM is inhibited. Therefore, since there is only clear evidence for gene targets of the Notch signalling pathway to oscillate across the chick PSM, it is less problematic to ask whether somitogenesis can occur in the absence of this one oscillating signalling pathway in the chick.

Since it is known that the complete absence of Notch signalling abolishes the formation of somites in the mouse model (Ferjentsik et al., 2009), I wanted to know whether the same is true in the chick model. To this end, I performed an experiment whereby the Notch inhibitor LY, or the equivalent volume of DMSO, was added *in ovo* to developing chick embryos at the point of gastrulation (HH4, roughly 17-18 hours post-fertilisation) and they were left to develop *in ovo* until HH10 or 11 (Figure 2.3 A). Since the inhibitor has a half-life of about 12 hours, it was reapplied after this time period, by which embryo had developed to HH8 (roughly 30 hours post-fertilisation), then incubated for a further 6 hours to HH10 or HH11, before the embryos were dissected, fixed, and analysed by *in situ* hybridisation for somite and PSM marker genes (*cParaxis* and *cTbx6* respectively). The somites developed normally in the DMSO-treated embryos, and this was reflected with the *cParaxis* and *cTbx6 in situ* hybridisation results (n=7/7) (Figure 2.3 B, F). The number of somites in the embryos from HH9-11 varied between 9 and 13, which is within the normal range for these stages (there was one younger embryo at around HH8 which produced 5 somites, which is normal for this stage). However, the embryos that were treated with LY displayed severely disrupted somite formation, although in all the embryos, several condensations were still being produced, they were formed very irregularly in size (n=7/7) (Figure 2.3 C, D, E, G, H, I). Of

these samples, 3/7 produced a stunted body axis; the number of somites was very hard to calculate in these samples, since the segmental boundaries were poorly formed and difficult to analyse. However, it can be estimated that the number varied from 3 to 7 in 6/7 embryos (1/7 had 9-10 somites, which is normal for HH10). Because the embryos were at roughly the same stage as their DMSO-treated counterparts (as judged by their head development), this was considered to be a much lower number of somites. This demonstrates that Notch signalling is required for normal somite formation in chick embryos.


A
LY411575
HH4
Overnight
HH7/8
LY411575
6 hours
Dissect and fix at HH10/11

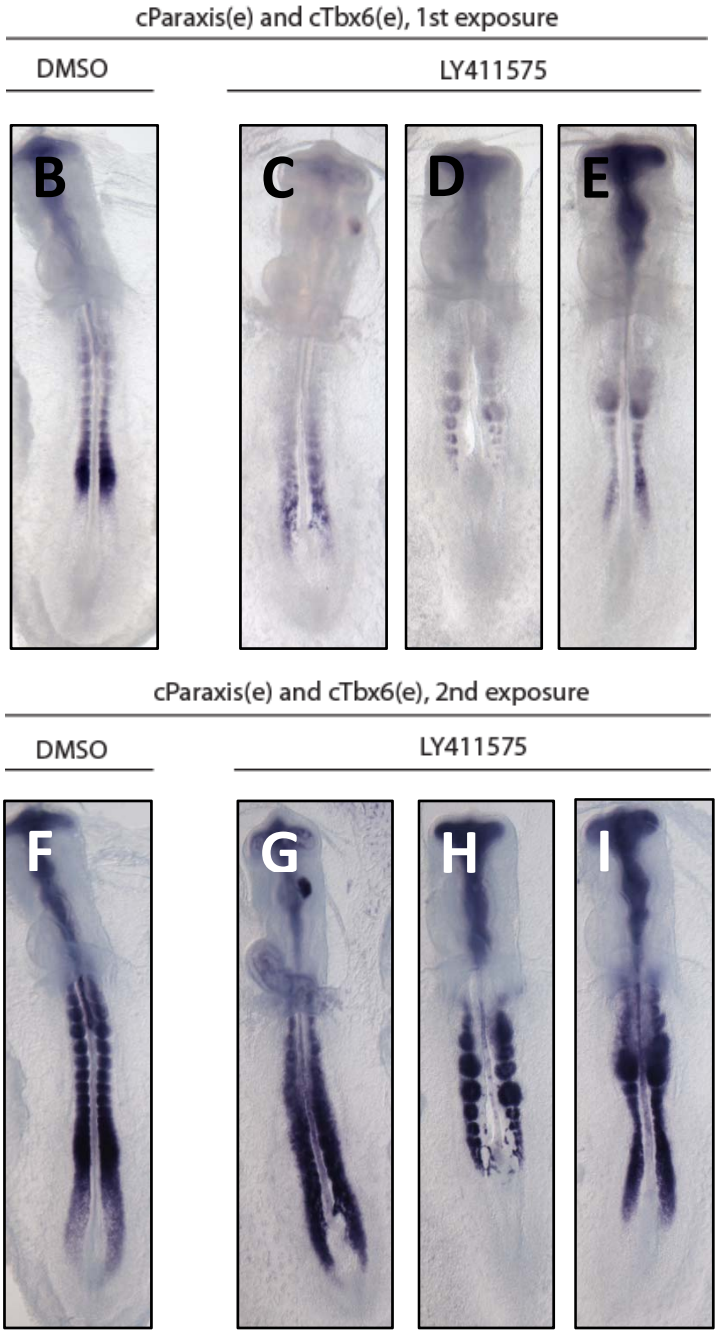

cParaxis(e) and cTbx6(e), 1st exposure
DMSO
LY411575
B
C
D
E
cParaxis(e) and cTbx6(e), 2nd exposure
DMSO
LY411575
F
G
H
I

Figure 2.3 *In ovo* addition of LY411575 to chicken embryos disrupts somitogenesis

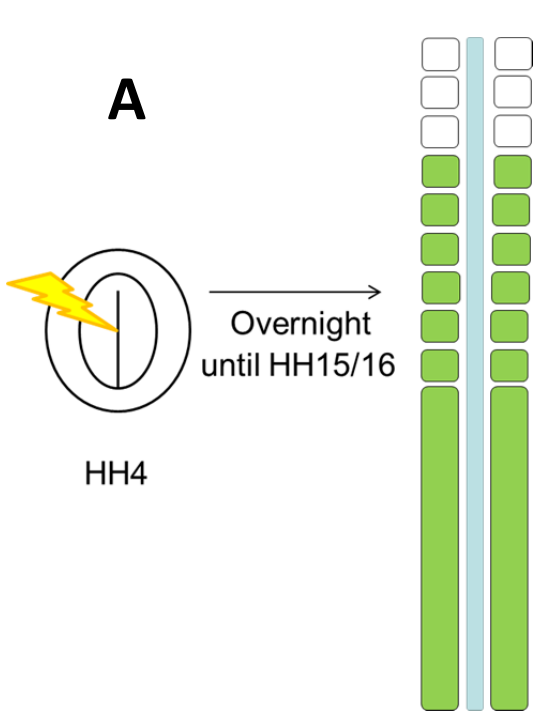
A) The assay. B-I) Dorsal view of Chicken embryos cultured *in ovo* with 150 nM LY411575, or DMSO then harvested and analysed by in situ hybridisation for *cParaxis (e)* and *cTbx6(e)*. The embryos were imaged at two time points: the first exposure, where only cParaxis had been detected (B-E) and the second exposure, where cTbx6 had also been detected (F-I). Control embryos produced normal somites (B, F), LY-treated embryos formed abnormal and appeared more like condensations than proper somites (C, D, E, G, H, I).

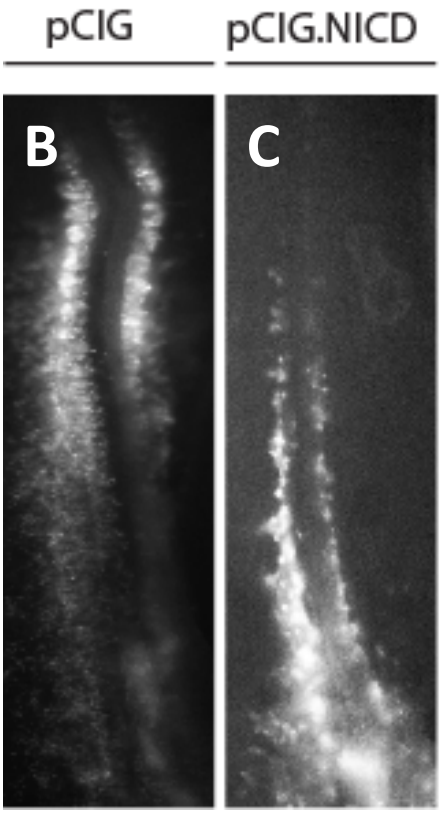
Electroporation of NICD into PSM precursor cells disrupts somitogenesis in chicken embryos

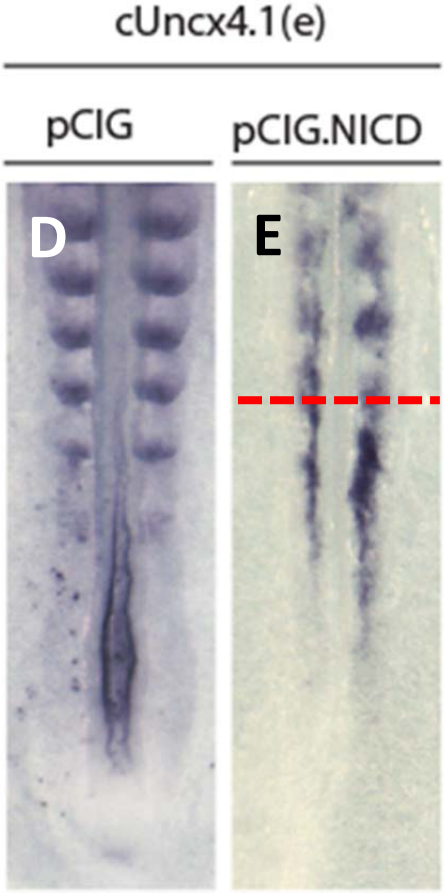
Since I have shown that Notch signalling is required for somitogenesis in the chicken embryo *in ovo*, the next question to ask was whether disrupting the oscillations of Notch signalling also affected the formation of somites. Previous studies have shown that electroporating a plasmid that codes for the active component of the Notch receptor (NICD) into the PSM of chicken embryos disrupts the cyclic activity of the Notch target *cLfng* (Dale et al. 2003). However, the effect of electroporating NICD on the formation of somites has not yet been analysed. To this end, we repeated the *in ovo* electroporation experiment of either the plasmid containing the NICD-coding gene and GFP (pCIG-NICD) or a plasmid only containing GFP (pCIG) into the PSM precursor cells in the primitive streak (PS) of HH4/5 chicken embryos (located at position 70% of the PS). Following this electroporation, the embryos were incubated for 24 hours until the embryos reached HH15 or HH16, before the embryos were harvested (Figure 2.4 A). At this point we were able to observe that the plasmid had been incorporated into the PSM and the first 5 to 6 pairs of somites (Figure 2.4 B, C), which would allow me to ask whether the presence of constant Notch activity had affected the formation of somites.

Following the collection of these embryos, they were analysed by *in situ* hybridisation for the gene *cUncx4.1*, which is both a marker of the caudal somitic compartment, and is also a target of Notch signalling. I therefore expected to see an up regulation of *cUncx4.1* expression where we see expression of the plasmid, as this indicates the presence of sustained Notch signalling activity in the somites. The results showed that while in the pCIG-electroporated control embryos the expression of *cUncx4.1* was restricted to the caudal compartment of somites (Figure 2.4 C) (n=2), in the pCIG-NICD-electroporated embryos the

somite boundaries were much less defined and expression of *cUncx4.1* was much more ubiquitous, and was often detected in the rostral half of the affected "segments" (Figure 2.4 D) (n=1). When we took transverse sections through the embryos we were able to show that the cells that express *cUncx4.1* in the rostral region of the somites (where *cUncx4.1* is not normally expressed) (Figure 2.4 E) also express the NICD plasmid (Figure 2.4 F). This shows that the expression of constant levels of Notch activity (and therefore all cyclic activity) in the chicken PSM disrupts the formation of somites, and potentially affects the rostro-caudal identity of the somites. Disruption of segmented region is progressively more severe in more caudal region. This is to be expected since there will be a developmental delay between the time the tissue first expresses the plasmid and the time when the protein product of this construct can elicit its effects. Moreover those effects will be most noticeable in the tissue exposed high NICD at a time when it is still in the caudal non-determined region of the PSM.


A
Overnight
until HH15/16
HH4


pCIG
pCIG.NICD
B
C


cUncx4.1(e)
pCIG
pCIG.NICD
D
E

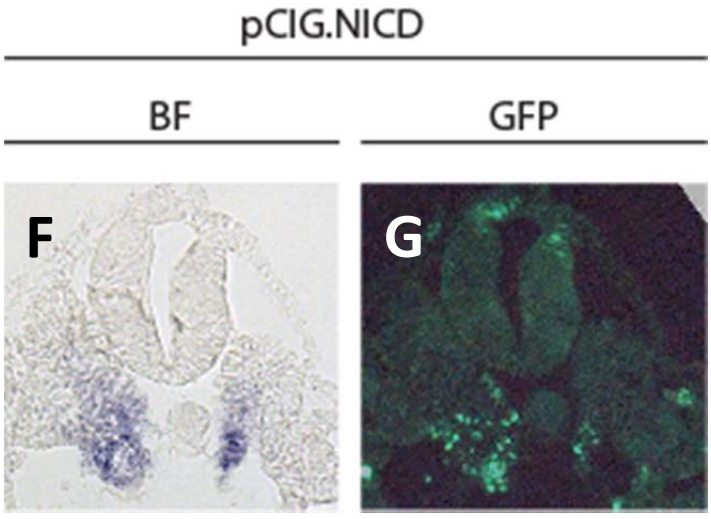

pCIG.NICD
BF
GFP
F
G

Figure 2.4 Constant Notch activity disrupts somitogenesis in the chicken embryo

Dorsal view of Chicken embryos electroporated at HH4 or HH5 with a GFP-expressing plasmid either with or without an NICD-encoding gene, and analysed at HH15 or HH16 (B, C). *In situ* hybridisation with *cUncx4.1* reveals that in the pCIG-electroporated embryos, cUncx4.1 is detected in the caudal compartment of somites (C), whereas in the embryos electroporated with pCIG-NICD, *cUncx4.1* expression is much more ubiquitous in affected segments (D). Sectioning through the region of unusual *cUncx4.1* expression in a rostral somite compartment (indicated by the red dashed line) reveals a consistent overlap of *cUncx4.1* and GFP expression (E, F). BF= Bright Field.

Chapter 3: The Role of MicroRNAs in Somitogenesis

Introduction

Many studies have shown that disruption of somitogenesis and the segmentation clock results in skeletal defects later on in vertebrate development. The segmentation clock causes the periodic expression of "clock" genes which cycle across the PSM with a periodicity that matches somite formation. The oscillation of these genes requires that the mRNA transcripts have short half-lives, which are rigidly coordinated during the oscillations. The mechanism for which the stability of these mRNA transcripts is controlled is currently unknown, but it is possible that it could be regulated by miRNAs, and that disruption of miRNA activity could be responsible for the skeletal defects observed in several syndromes.

In order to investigate this possibility, a mutant mouse line was generated in which the miRNA-processing enzyme Dicer was absent in the PSM tissue. This was established by crossing a T(s)::Cre transgenic mouse, in which the Cre expression is driven by the Brachyury (T) promoter in the PSM; with Dicer fx/fx and Dicer +/- mice. Dicer+/-; T(s)::Cre males were then mated with Dicer fx/fx females to produce Dicer fx/-;T::Cre mice which would lack Dicer activity in the PSM. This conditional knock-out model caused the embryos to survive until birth, thus allowing us to observe the effects of the loss of miRNA activity on somitogenesis.

Using this model, we set out to characterise the skeletal defects that arose from the conditional mutation by allowing the mouse embryos to develop to stages where the

skeletal structure could be examined. Following this, we established the link between loss of miRNA function and the segmentation clock by examining the behaviour of clock genes in the PSM of Dicer fx/-;T::Cre mice.

The Phenotype of PSM-Conditional Dicer mutant

In order to observe what effect the absence of miRNA activity in the PSM had on the development of the axial skeleton, embryos which had developed to E16.5 were dissected, and Alcian blue and Alizarin red were used to stain the cartilage and bones of the embryo respectively (Figure 3.1). This staining technique revealed severe segmental defects which had arisen in the mutant embryos. The regions of the mutant mouse skeleton which showed these malformations were the thoracic, lumbar and sacral regions, in which there were many irregular and fused structures: in wild-type embryos there are thirteen thoracic vertebrae and thirteen ribs (Figure 3.1 A) ; however, in the mutant embryos, there appears to be an extra rib (r14), which indicates that there is another thoracic vertebra (T14), perhaps formed at the expense of the first lumbar vertebra (L1) (n=8) (Figure 3.1 D). Another observation made was that whereas the wild-type embryos possess six lumbar and four sacral vertebrae, the mutant embryos possess five lumbar and five sacral vertebrae (Figure 3.1 E).

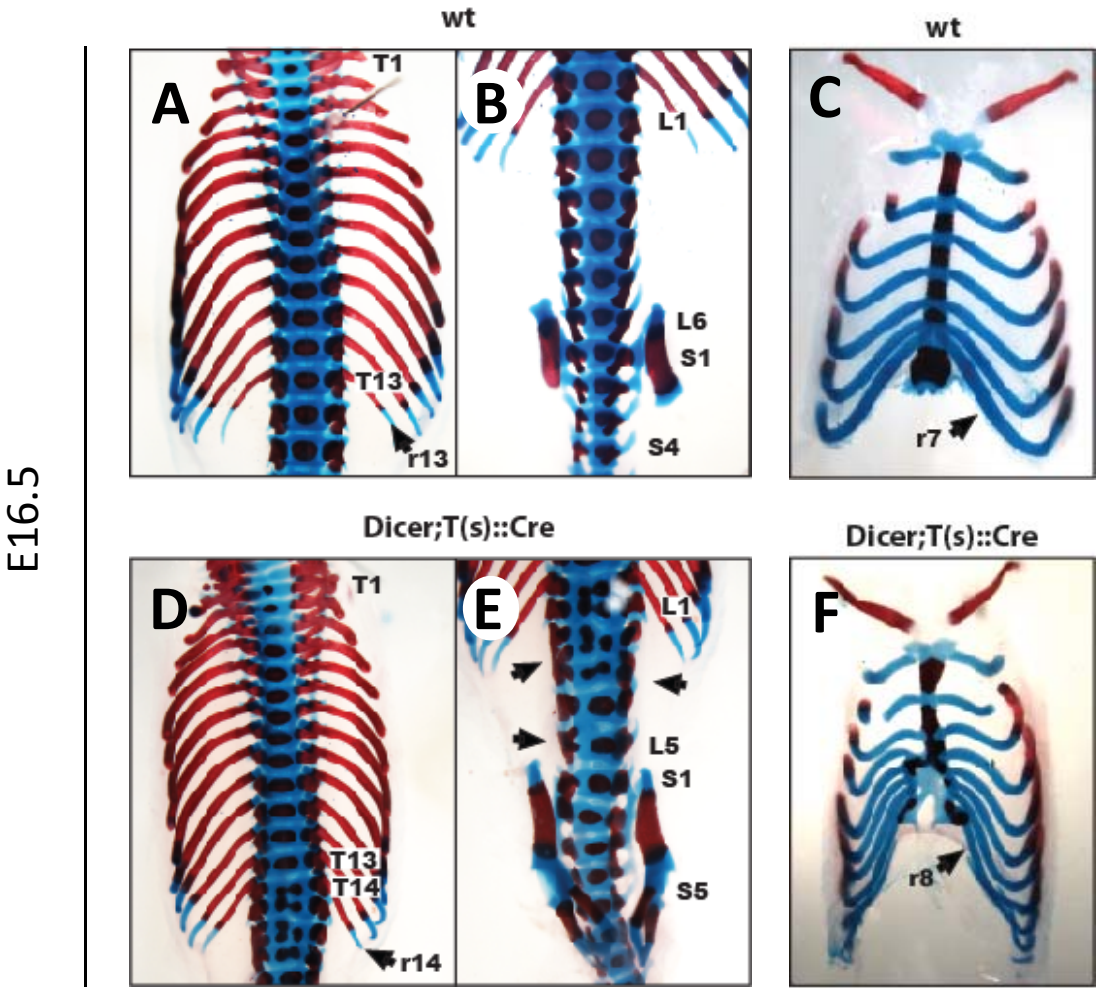

E16.5
wt
A
T1
T13
r13
B
L1
L6
S1
S4
wt
C
r7
Dicer;T(s)::Cre
D
T1
T13
T14
r14
E
L1
L5
S1
S5
Dicer;T(s)::Cre
F
r8

Figure 3.1 Dicer-mutant embryos have deformed lumbar and sacral vertebrae, and produce an extra pair of ribs

Wild-type (wt) and Dicer fx/-;T::Cre mice were collected at E16.5, followed by Alcian blue/Alizarin red staining of cartilage and bone. Wt embryos have thirteen thoracic vertebrae, thirteen pairs of ribs (A) (7 of which are attached to the sternum (C)), six lumbar vertebrae and four sacral vertebrae (D). The Dicer fx/-;T::Cre embryos display irregular and fused vertebrae: there is an extra thoracic vertebra (T14), as well as an extra pair of ribs (r14) (D) which is attached to the sternum (r8) (F). The last lumbar vertebra (L6) is also replaced by another sacral vertebra (S1) (E).

The lack of miRNA activity does not affect the segmentation clock in E9.5 and E10.5 embryos

Since the malformations are most prominent in the lumbar and sacral regions, derived from somites 25-34, we next observed the effect of miRNA inactivity in the PSM of mouse embryos in which these somites were being generated. E9.5 and E10.5 wild-type and Dicer fx/-;T::Cre mouse embryos were subjected to *in situ* hybridisation for *mUncx4.1*, a marker of the caudal half of somites, to observe how the new somites were generated. In E9.5 mice, in which 20 to 26 somites had been formed, there was no obvious malformation in the nascent somites (Figure 3.2 A, B). However, in E10.5 mice, which have between 34 and 40 somites, some of the most recently formed somites begin to show severe defects (Figure 3.2 D, E) when compared to the wild-type embryo (Figure 3.2 C). The defects extend over a distance of 9 or 10 somites, before somitogenesis recovers, and the affected area is only found caudal to somite 25/26. This affected region corresponds to the vertebral defects observed in the skeletal phenotype observed in E14.5 and E16.5 mice, showing that those skeletal defects arise from improper somitogenesis which begins at the formation of somite 25 and returns to normal after somite 34.

Since the Dicer-mutant mice display skeletal defects, it is logical to assume that these defects could have arisen during the process of somitogenesis, so I also decided to investigate this possibility. The segmentation clock is thought to be crucial for somitogenesis, therefore I wanted to ask whether the oscillations of clock genes was disrupted in the PSM of mutant embryos. Because the mRNAs and proteins involved in the clock often have short half-lives, it is possible that miRNAs are responsible for this, and the loss of miRNA function may lead to disruption of the segmentation clock.

The embryos which had been analysed for *mUncx4.1* expression were also hybridized for *mHes7* RNA in the PSM of both E9.5 (n=10) and E10.5 (n=5) embryos to observe whether the oscillations still took place (Figure 3.2). For the E9.5 mice, there were distinctly different phases of *mHes7*: three embryos were in Phase 1 (Figure 3.2B); three embryos were in Phase 2 (Figure3.2A); four embryos were in Phase 3 (not shown). For E10.5 mice, there were also distinctly different patterns of *mHes7* expression: two embryos were in Phase 1 (Figure 3.2D; one embryo was in Phase 2 (Figure 3.2E); two embryos were in Phase 3 (not shown). This shows that the segmentation clock is not disrupted in the mutant embryo PSM, and that the lack of miRNAs must be affecting a different component of somitogenesis.

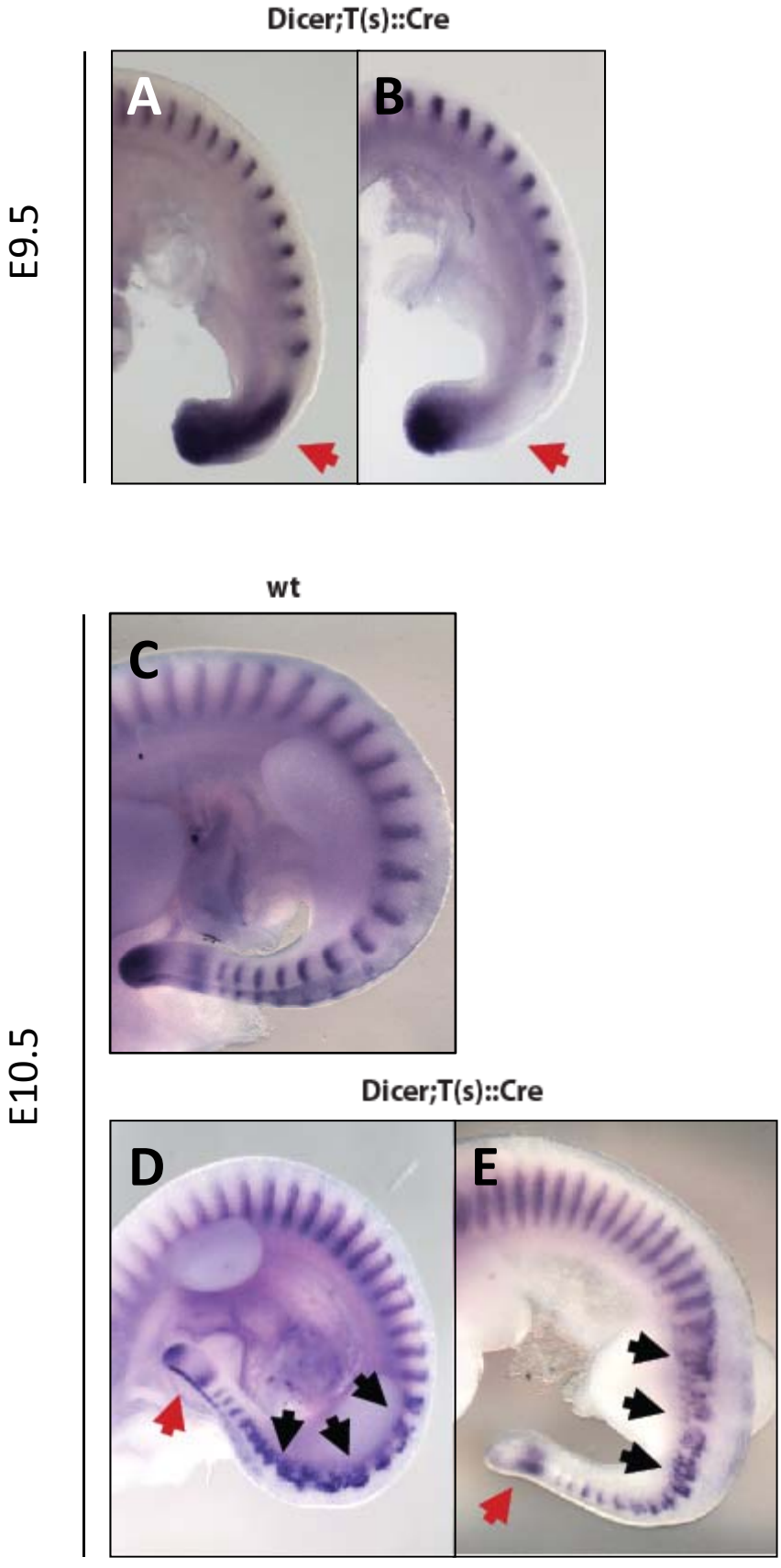

Dicer;T(s)::Cre
A
B
E9.5
wt
C
E10.5
Dicer;T(s)::Cre
D
E

Figure 3.2 Somite defects are present at E10.5 in the Dicer mutant PSM, and Hes7 expression is unaffected

Expression of *mUncx4.1* and mHes7 in wild-type and Dicer fx/-;T::Cre mouse embryos was analysed at stages E9.5 and E10.5. In E9.5 mice embryos there was no evidence of any somitic defects and different phases of Hes7 were observed (red arrows) (A, phase 2; B, phase 1).

In E10.5 embryos, there are somitic defects as detected by disordered *mUncx4.1* expression over a 9-10 somite range (black arrows) (D, E), whereas the wild-type embryos displayed clearly defined *mUncx4.1* expression in all the somites (C). mHes7 expression is still dynamic in the PSM of Dicer fx/-;T::Cre embryos (red arrows).

Gene expression in the PSM of Dicer;T(S)::Cre embryos, and miRNAs expressed in the PSM

Identification of the genes which show a significant change in expression in the PSM of mutant embryos when compared to wild type embryos will be required to analyse the mechanism by which the skeletal defects found in these embryos arise. To this end, a gene expression analysis was performed using the Mouse GE4X44Kv2 Microarray from Agilent (IMGM laboratories). RNA was collected from the PSM and three somites from both control and Dicer-mutant embryos at E9.5. The results showed that there was a high correlation between the control and mutant groups, with only some minor differences. However, the genes which displayed minor differences, a total of 192 with a p–value ≤ 0.00001, were further analysed for association with biological processes (www.pantherb.org), and showed four categories which were enriched in mutant PSMs: "System Development", "Developmental process", "Skeletal system development" and "Mesoderm development". Interestingly, the genes which were up regulated include *Lfng*, which indicates that the segmentation clock may be affected, even though the expression profile of *mHes7* was unaffected in E9.5 and E10.5 mutant embryos. Several 'posterior' *Hox* genes were also shown to be up regulated, such as Hoxc8 and Hoxb9. Since *Hox* genes specify the positional identification of the vertebrae, changes in the amounts of specific *Hox* genes that are regulated by miRNAs could potentially change the positional identity of the vertebrae as they form. This supports the idea that specific posterior vertebrae identity is being affected by the lack of miRNA activity in the PSM (data not shown).

In a complementary approach I set out to identify the miRNAs which are present in the PSM, as this will help determine which specific miRNAs may be responsible for modifying gene expression in this tissue. According to MicroRNA.org, there are 717 miRNAs expressed in the

mouse embryo, so in order to identify which miRNAs are expressed in the PSM, a miRNA profiling screen was performed (Source BioScience LifeSciences) using a TaqMan Mouse microRNA Assay Set (Applied Biosystems). To prepare for this screen, pooled samples of PSM/tailbuds were collected from E9.5 embryos, and the levels of miRNA expressions were compared with those of the whole embryo. The screen allowed the identification of several miRNAs which are highly expressed within the PSM (not shown): interestingly, two of these miRNAs, miR-10a/b and miR-196a/b, have been previously shown to regulate *Hox* gene expression (He et al., 2011; McGlinn et al., 2009; Yekta, Shih, & Bartel, 2004).

To test whether the expression of these miRNAs in the PSM corresponds to this data, *in situ* hybridisation was performed using Locked Nucleic Acid (LNA) probes to detect expression in the PSM (Figure 3.3). The data from this in situ hybridisation confirmed the presence of miR-196a in the PSM, which indicates that the lack of this miRNA in the Dicer-mutant embryos may be the cause of the observed skeletal defects, possibly due to the dysregulation of specific *Hox* genes.

Figure 3.3 MiR-196a is expressed in the PSM of E9.5 mice embryos

Lateral view of wild type E9.5 embryos showing the expression of miR-196a in the tail region as detected with a LNA probe using *in situ* hybridisation.

Discussion

Dll1(i) transcripts oscillate in the mouse PSM

The first part of the work which has been carried out in this thesis shows that transcriptional activation at the promoter of the Notch pathway ligand *Dll1* is oscillatory in the mouse PSM in the manner of a clock gene. This is the only ligand of the Notch family to display this cyclic expression in the PSM of either the chick or mouse embryo. This is an important finding as it indicates that the expression of *Dll1*, which is crucial for canonical Notch signalling, is regulated by the segmentation clock. Since it has previously been shown that there is cyclic expression of NICD, which was first detected through staining by immunohistochemistry (Huppert et al., 2005) and which in turn causes cyclic activation of Notch target genes, this could mean that the cyclic levels of *Dll1* are responsible for these downstream events.

A curious finding is that we only see cyclic expression of the intronic mRNA transcript for *mDll1*, rather than the mature mRNA transcript. This means that while there is dynamic transcription of *mDll1(i)*, the mature transcripts are relatively stable when detected by *in situ* hybridisation. This does not mean, however, that there is no dynamic expression of the mature mRNA transcripts, and perhaps our method of detection is not sensitive enough to recognise this. Moreover, the presence of stable mature mRNA transcripts does not necessarily reflect active translation of the proteins: a next goal of this project would be to establish an assay which would determine whether the mature transcripts were being actively translated. It has also been shown in recent unpublished data in the lab that the intronic mRNA transcripts for the receptor Notch1 also oscillate in the PSM of both mouse

and chick embryos, whereas the mature transcripts do not. Moreover it was shown this *Notch1(i)* expression itself is regulated by the Notch pathway, as proven by inhibition of Notch signalling using LY in explant culture, which completely down regulates *Notch1(i)* transcription. It is also significant that the transcripts for *mNotch1(i)* and *mDll1(i)* oscillate out of phase with one another following 'culture-culture' assays, which further strengthens the argument that *mDll1(i)* oscillates out of phase with Notch targets.

The cyclical nature of both the *Notch1* and *Dll1* nascent mRNA transcripts indicates that there may also be oscillations of Notch1 and DLL1 proteins in the PSM. We have recently looked at the expression of these proteins using monoclonal antibodies, and using immunohistochemistry to observe their spatial distribution in the PSM of the mouse embryo (Appendix 1). When viewing the whole tail, it appears that there are no oscillations of these proteins in the PSM, since different embryos display a very similar expression profile which is manifest as a rostro-caudal gradient. However, because the interactions between Notch1 and DLL1 in the PSM are very sensitive to small fluctuations a higher resolution analysis is required to determine if minor fluctuations of proteins do occur, which would explain minor fluctuations of nascent mRNA transcripts. This detailed analysis of Notch and Delta protein expression is underway. Notch and Delta proteins on neighbouring cells bind to each other in order to activate Notch signalling. This interaction is known as *trans*-activation, and results in the proteolytic cleavage of the intracellular domain of the Notch receptor in the receiving cell. However, there is a second type of interaction, which arises when the Notch and Delta proteins from the same cell interact, known as *cis*-inhibition, which causes the inactivation of Notch receptor cleavage, thereby inhibiting Notch signalling in that cell. The ratio of Notch and DLL1 proteins in each cell within the PSM is therefore very important to

the propagation of Notch signalling in a unilateral direction in the PSM, and small fluctuations of these ratios affects an ultra-sensitive switch in these cells which alternates between a mutually exclusive sending or receiving state (Sprinzak et al., 2010). Due to the sensitivity of this switch, small differences in the amount of Notch1 and DLL1 proteins would theoretically have a large effect on the signalling state of the cell, and the link between the regulation of Notch and Delta proteins and transcriptional oscillations of their mRNA transcripts remains to be investigated. Moreover, both Notch and Delta proteins are subjected to a huge amount of trafficking to and from the plasma membrane for a variety of purposes (to be trafficked to the lysosomes for degradation, to be activated in the case of Delta, or for Delta-Serrate-Lag2 (DSL) independent Notch activity). Consequently, subcellular localisation of the receptor and ligand within this gradient expression domain will also be highly informative.

Using these antibodies, we will first ask whether there is a difference in the ratio of where full length Notch1 and DLL1 proteins are located in the plasma membrane of a single cell, and if so whether this ratio differs across the PSM. By addressing this question we would hope to determine whether there is a dynamic fluctuation of *cis*/*trans* activity that acts throughout the PSM, and whether this would be responsible for the dynamic oscillations of cyclic gene targets of the Notch pathway. In these experiments, the NICD antibody would hopefully serve as a reliable readout of *trans*-activation within the PSM. If our predictions are correct, then a pattern of Notch *trans*-activation would occur in a caudo-rostral direction through the PSM, and would correspond to regions of NICD expression. If this proves to be the case, then this would explain how oscillations in the PSM are propagated in a unilateral manner in the PSM.

mDll1(i) oscillates as Wnt target, and requires Notch and Wnt signalling to be expressed

We also demonstrated that *mDll1(i)* oscillates out of synchrony with Notch signalling, and in synchrony with the Wnt target *mSnail1(i)*, indicating that *mDll1(i)* oscillations are regulated by the Wnt pathway. This was confirmed when drug culture experiments revealed that *mDll1(i)* is down regulated in the absence of Wnt, in accordance with previous data (Juan Galceran et al., 2004; Hofmann et al., 2004). However, we have also shown that there is a role for Notch signalling in the transcription of *mDll1(i)*, since its expression is only completely abolished when Notch signalling is down regulated concomitantly with Wnt signalling. These data establish *mDll1(i)* as a new point of cross-talk between the Notch and Wnt pathways, and could be an indication that Wnt partially regulates the initiation of Notch signalling through the transcriptional activation of *mDll1* in the PSM. In future studies, we would also like to take a more quantitative approach to examining the effect of Notch and/or Wnt inhibition on the expression of *mDll1(i)* using techniques such as qPCR. In these experiments we would gather the total RNA from a treated explant, before performing reverse transcription to generate cDNA from these samples and use primers for *mDll1* to perform qPCR, which would allow us to quantify the level of down regulation of the *mDll1(i)* transcripts following drug inhibition.

We would also like to take this analysis further and observe the effects of inhibiting Notch and/or Wnt signalling *in vitro* using primary cells from the PSM, as well as C3H10T1/2 fibroblasts. In these experiments we would analyse the nature of expression of the 4.3kb Dll1 promoter construct and determine its dependency on Notch and Wnt signalling

pathways using the same inhibitors used in our experiments (i.e. LY for inhibiting Notch, XAV for inhibiting Wnt). The construct we would wish to use was first described in a paper from the group of Professor Ryochiro Kageyama, in which the 4.3kb promoter for Dll1 was cloned upstream of a ubiquitinated firefly luciferase reporter. This study revealed *in vivo* and *in vitro* that oscillating Dll1 expression occurs in neural progenitor cells, but that stable expression of Dll1 persisted in cells which had begun neuronal differentiation (Shimojo et al., 2008). This method was first described in an earlier paper from Kageyama's group, where the cyclic expression of Hes1 was analysed using real time bioluminescence techniques, both in the PSM of transgenic mice and in dissociated PSM cells and C2H10T1/2 mouse fibroblast cells (Masamizu et al., 2006). The use of luciferase assays would help us to determine a more quantitative approach to realising the dependency of *mDll1* transcription on both Notch and Wnt signalling. The luciferase readouts would then be quantified using OMERO™ software.

Hes7 regulation of *mDll1* in the caudal PSM

We also show evidence that there is a role for Hes7 in the regulation of *mDll1*. This was shown in the Hes7-/- mice, which lack the Hes7 protein, thereby maintaining constant levels of Notch activity in the PSM. The mRNA profile for mDll1 was very unusual because whilst expression levels were generally up regulated in the PSM and most recently formed somites, its strongest domain of expression was located in the posterior PSM. In this region of the PSM, it has been show that the expression of Hes7 is dependent on the FGF pathway, rather than the Notch pathway (Niwa et al., 2007). In this paper, it is thought that the initiation of Hes7 oscillations is regulated by the FGF pathway, while the propagation and amplification of Hes7 expression across the PSM is under the control of Notch signalling. If we add our data to this model it would suggest that Hes7 negatively regulates *mDll1(i)* expression in the tail bud; once Hes7 protein degrades, *mDll1(i)* transcription would be initiated in the tail bud, which in turn would initiate oscillations of Notch signalling in the PSM. The activation of Notch signalling in the cells then allows the transcription of Hes7, which then causes the cycle to repeat itself. Thus the three main pathways, Notch, Wnt and FGF, would be involved in complex feedback loops which allow the initiation of cyclic gene oscillations to occur in the PSM.

This model, however, requires further experiments in order to confirm it. Because we have relied on the kind contributions of pre-fixed Hes7-/- embryos from Yasumasa Bessho's group, we have been unable to perform culture experiments with these embryos ourselves. Since we are hoping to establish our own line of Hes7-/- mice in the near-future, this would allow us to perform some previously impossible experiments. Firstly, we would be able to analyse the cyclic behaviour of *mDll1(i)* in more detail using 'fix and culture' experiments,

where the tails of E10.5 Hes7-/- embryos would be halved and cultured for different time periods (one explant would be cultured for an hour longer than the other), before analysis with *in situ* hybridisation. This would allow us to determine if the fixed pattern of expression which is observed in wholemount tails means that oscillations of *mDll1(i)* have ceased in the presence of constant Notch activity. It would also be possible to further investigate the role of Hes7 protein in inhibiting the transcription of *mDll1*, as our data so far indicates that the down regulation of Hes7 allows the overexpression of *mDll1(i)* in the tail, specifically in the posterior PSM, but we do not yet know whether Hes7 directly suppresses the transcription of *mDll1(i)*, or whether this occurs via the action of another downstream target of Hes7 repression. I would firstly aim to observe the expression profile of the Hes7 protein in the mouse PSM, and see whether there was mutual exclusivity between the presence of Hes7 protein and *mDll1(i)*. If there is, then this would strengthen the argument that Hes7 represses *mDll1(i)* transcription in vivo. We could then further test this idea by performing chromatin immunoprecipitation (ChIP) on mouse embryo tails to see whether there is a direct interaction between the Hes7 protein and the mDll1 promoter region. If there are Hes7-binding sites in the promoter region, then a series of experiments could be performed whereby the promoter region of mDll1 was cloned into a reporter construct containing firefly luciferase. The binding sites for mHes7 could then be mutated, and subsequent analysis of the readout of luciferase would confirm the role of Hes7 in regulating *mDll1(i)* (i.e. if the luciferase signal is stronger in the mutated Hes7-binding site construct, this would further substantiate the idea that Hes7 supresses *mDll1* transcription in the tail bud). This approach has previously been adopted to observe the regulation of *mDll1* in the PSM, and has shown that *mDll1(i)* relies on Wnt signalling both directly through Wnt transcription

factors, and indirectly through its downstream target Tbx6 (Galceran et al., 2004; Hofmann et al., 2004).

We can also test our theory that Hes7 negatively regulates *mDll1* transcription using pharmacological inhibitors in explant culture experiments. Since FGF signalling regulates expression of Hes7 in the tail bud, by inhibiting FGF expression this would abolish *Hes7* mRNA and protein from this region of the PSM (Niwa et al., 2007). If our model which states that Hes7 regulates *mDll1(i)* in this region of the PSM is accurate, then the lack of Hes7 in the tail bud due to lack of FGF signalling could then reproduce the expression profile of *mDll1(i)* that we observed in the Hes7-/- mice.

We also observe in the Hes7-/- mice that *mDll1(i)* is expressed in the most newly formed somites. Since *mDll1(i)* is not normally expressed in this region of the embryo, this could also indicate an interesting role for Hes7 negatively regulating Notch signalling in nascent somites. This was an unexpected finding, as Hes7 protein is not normally detected in the somites. Even though the expression of Mesp2 still persists in the Hes7-/- mice, its boundaries are less well-defined than in wild-type embryos (Ferjentsik et al., 2009), which is also seen in other Notch mutant PSMs, and indicates that non-cycling Notch activity in the PSM will result in faulty somite boundary formation, and could account for the ectopic expression of *mDll1(i)* in the somites.

Are oscillations required for somitogenesis?

In transgenic mice with up regulated Wnt pathway effector molecule β-catenin, it has been shown that increased levels of β-catenin has no effect on the oscillations of the cyclic genes *mNkd1* and *mLfng* (Aulehla et al., 2008), and the up regulation of Wnt by Lithium chloride (LiCl, an inhibitor of GSK3β, and therefore a Wnt agonist) or Wnt3a-conditioned media also does not affect the oscillations of the Notch target *cLfng* in the chick PSM (Gibb et al., 2009) and *mLfng* in the mouse PSM (unpublished data). However, in the transgenic β-catenin mice, the levels of β-catenin were shown to be massively up regulated, which in turn massively up regulate Wnt signalling activity, as evidenced by the increased level of expression for the Wnt target gene *mAxin2* (Aulehla et al., 2008). Since we aim to determine the effect of constant (but not up regulated) Wnt activity across the PSM on clock gene oscillations, this mutant mouse is likely to have a different effect to constant levels of LEF1, which is what occurs in the mouse model we are using.

Although the study in which LiCl or Wnt3a media was added to the mouse explants in culture was used to examine the oscillation of the Notch target *mLfng*, the oscillations of Wnt and FGF target genes was not reported. Because we observe the continuation of *mLfng* oscillations in the CatC-Lef1 mouse PSM, using Wnt3a-conditioned media or LiCl would theoretically cause the same expression profiles of Wnt targets in the PSM that we observe in CatC-Lef1 mutant mice, and therefore should be repeated. However, because the use of LiCl or Wnt3a-conditioned media would act to upregulate Wnt activity instead of stabilising Wnt oscillations, it is possible that we would see the same effects as for the β-catenin transgenic mice, i.e. no difference in the cyclic gene oscillations. However, this remains to be investigated.

It could be the case that in a system where Wnt/FGF oscillations are halted, Notch oscillations continue unimpaired, thus providing a mirror image of the situation in the Hes7-/- embryos. If the hypothesis that somitogenesis requires oscillations of cyclic genes in the PSM is accurate, then we would expect the oscillations of Wnt/FGF targets alone to be sufficient for somite formation. Once these two models have been investigated, we would then be able to cross them to generate a mouse with a background in which there are no oscillations of any cyclic genes, and we could thus see whether somitogenesis can occur in these conditions.

Despite the work that has been shown to highlight how the disruption of the components of somitogenesis (and in particular components of the Notch signalling pathway) causes vertebral defects to be formed, the origin of these disorders remains difficult to classify. However, recent work has established a link between environmental causes of skeletal defects and disrupted somitogenesis. Congenital Scoliosis (CS) causes lateral curvature of the spine exceeding 10°, caused by improper skeletal formation, but has a wide range of phenotypes, and affects one in every thousand live births. It is therefore more common than SCD and STD, and is distinct from these recessive monogenic traits which only affect the vertebrae and are therefore easier to classify (Sparrow et al., 2011). It did not appear likely that CS would have a simple genetic aetiology. However, a recent study has shown that by exposing a pregnant mouse to short-term hypoxic conditions, the penetrance and severity of vertebral defects are increased in embryos which are haplo-insufficient in Notch pathway genes Mesp2 or Hes7. When the E9.5 embryos were analysed following hypoxia, it was revealed that Hes7 protein levels were abolished, accounting for a loss of cyclic NICD expression in the PSM (instead NICD expression is stable, with a prominent rostral band);

also in these embryos, the expression of the normally dynamic pERK (FGF effector molecule) is abolished, which explains why Hes7 fails to be expressed. The model generated in this paper stated that treatment with hypoxia caused skeletal defects in the embryos by primarily disrupting FGF signalling in the PSM, and by subsequent disruption of Notch and Wnt signalling (Sparrow et al., 2012). Because we have the resources to generate hypoxic conditions in explant culture experiments, it would be interesting to further explore the effects of low oxygen conditions on somitogenesis, and observe how the segmentation clock is affected in greater detail. The study also showed that there was no difference between the levels of full-length Notch 1 or DLL1 proteins throughout the PSM. In relation to the *cis*/*trans* experiments mentioned above, it would be interesting to see if the constant levels of NICD in the PSM correlate with expanded regions of *trans*-activation.

Interestingly, when *Fgf4* and *Fgf8* are conditionally deleted in the PSM, the expression of most PSM-specific genes, including cyclic genes such as *Lfng* and *Hes7*, are abolished, and premature differentiation occurs in the PSM. It was also subsequently shown that at the late head fold (LHF) stage, which occurs just prior to the onset of somitogenesis, that *Lfng* is expressed throughout the PSM, due to the premature maturation of the PSM (Naiche et al., 2011). This would indicate that segmentation still occurs without the oscillations of cyclic genes; however this also is reminiscent of the phenotype observed in *Lfng-/-* mice, where somites are still formed, although they are irregular in size and are occasionally fused together (Ferjentsik et al., 2009). It remains to be seen if other cyclic genes such as *Hes7* still cycle in this genetic background, as well as pathway effectors i.e. NICD and pERK. Although this data is of great importance, it does not answer our question, because we aim to determine whether somites form in the absence of cyclic gene expression due to

constitutively active Notch, Wnt and FGF signalling. By depleting FGF signalling in the PSM, they have caused the premature differentiation of the PSM tissue, whereas we want to keep the PSM in an undetermined state to observe the effects of constant Notch, Wnt and FGF activity on somitogenesis.

Notch signalling and its oscillations are required for somitogenesis in the chick

The next step for our mouse model will be to generate a new mutant line of mice that will have a Hes7-/-;CatC-Lef1 background, which will in theory have no oscillatory targets of Notch or Wnt signalling, and will hopefully allow us to answer the question of whether oscillation of cyclic genes are required to generate somites. However, such a process is long and expensive, so concomitantly we decided to look in a simpler vertebrate model to partially answer this question: does disruption of Notch cyclic activity in the chicken PSM interfere with somitogenesis?

Since in the chick the only signalling pathway to have oscillation of target genes demonstrated to cycle across the PSM is the Notch pathway, this made it easier to eradicate cyclic gene expression in the PSM. However, the use of transgenic analysis is limited in the chick compared to the mouse model, so to combat this I adopted a different experimental approach: the first step was to establish the effects of long term Notch inhibition *in ovo* upon somite formation; following this we decided to establish the effects of constant Notch activity, and therefore disruption of cyclic gene expression, on the process of somitogenesis.

When we used the small molecular inhibitor LY to inhibit Notch signalling *in ovo*, in all 7 samples there was normal head development, however in these embryos there was a variety of defective axial phenotypes: in 3 out 7 embryos there was a shortened axial length, whereas the rest had normal length of axes; in all LY-treated embryos the somites were inconsistent in size and number, and the degree to which they had malformed varied. The number of somites was generally lower than the DMSO-treated embryos (with an average

of 10.4 somites from 7 samples), although it was sometimes very difficult to make out segmental boundaries. Because we see a range of severity in these phenotypes, and that segmentation still occurs in the absence of Notch signalling, that may represent a limitation of the technique implemented: because the drug is being added to the embryo *in ovo*, there is no guarantee that the drug is having a consistent effect in all of the treated embryos. An improvement to this procedure may be to inject the drug under the surface of the viteline membrane, thereby targeting the embryo more effectively. It can be said from these results, however, that the inhibition of Notch signalling severely disrupts somitogenesis in chick embryos.

Having established this, we then decided to observe the effect of constant levels of Notch activity throughout the PSM. Since this would prevent all cyclic activity in the PSM, would therefore allow us to determine the effects of halting the segmentation clock on the process of somitogenesis. To carry out this experiment, we implemented the technique of *in ovo* electroporation to transfect specific cells within the primitive streak which are precursors of the PSM and somites, with a plasmid expressing NICD. This is a technique which has been previously used to study the effects of up regulated Notch activity in the chick PSM on the cyclic expression of *cLfng*, and showed that cyclic gene expression had been abolished, and instead the level of mRNA had been up regulated throughout the PSM (Dale et al., 2003). However, in my experiments I allowed the embryos to develop until a much later stage HH15 or HH16, at which point the cells expressing the plasmid had been incorporated into the PSM as well as the first 5 or 6 pairs of somites, thereby allowing us to determine whether the presence of constant Notch activity affected formation of these somites. When analysed by in situ hybridisation for *cUncx4.1*, a marker of the caudal compartment of

somites, the expression profile was messy in comparison with that of embryos which had only been electroporated with the pCIG construct. The segmental boundaries were not clear, and there were no clear epithelial spheres to be called somites. *Uncx4.1* expression appeared across the whole rostro-caudal extent of the abnormally formed segments. When these embryos were sectioned, in the rostral area of these abnormal segments, regions of GFP expression (marking NICD expression) and *cUncx4.1* expression significantly overlapped, indicating that the presence of constant NICD expression causes the rostral compartment of the affected segments to become caudalised. It would be an interesting observation to allow these embryos to develop to a later stage when the segments had differentiated to form the vertebrae of the skeleton, and to see whether constitutively active Notch activity cause skeletal defects which resemble the skeletal phenotypes caused when Notch signalling is compromised or abolished. This would then convincingly argue that the presence of oscillations in the PSM is required for the correct formation of the axial skeleton.

However, we do observe some segmentation in the chick embryos which are electroporated with pCIG.NICD. There is some similarity here with the Paraxis-/- mouse, in which there are no somites formed but there is segmented myotome (Wilson-Rawls et al., 1999). This indicates that while the embryo is developing, somites are not essential for survival; however, without somites (and oscillations), the segmented structure of the body axis is very poor, and is incompatible with life.

The loss of Dicer in the mouse PSM causes skeletal defects

The conditional mutant mouse line for Dicer in the PSM has local somite irregularities, which lead to specific vertebrae defects later in development in the corresponding lumbar-sacral vertebrae which derive from the affected somites. Thus, this mutant mouse provides a unique tool with which to uncover profound insights into the role of miRNAs in somitogenesis. The effects of the loss of miRNA activity are most evident in the skeletons of mutant mouse embryos which have developed to a late stage (E14.5 and E16.5), showing deformities and fusions of several vertebrae. There was also the presence of an extra pair of ribs, indicating that the rate of segmental formation may have changed in the absence of miRNAs.

The phenotype observed in these embryos closely resembles that of chick embryos which were introduced to antagomiRs to block the activity of miR-196a during development *in ovo*: these embryos subsequently developed homeotic transformations, which included the transformation of the last cervical vertebra to a thoracic one (McGlinn et al., 2009). This also correlated with another study in which the knockdown of miR-196 in zebrafish caused the formation of an extra vertebra (He et al., 2011). Interestingly, miR-196 regulates several Hox genes, which indicates that the skeletal defects may be caused by the overexpression of Hox genes in the Dicer fx/-;T::Cre embryos. A transgenic mouse line which expresses Hoxc13 under the Dll1 promoter, thereby causing it to be overexpressed, also results in similar segmental defects as we have observed for the Dicer fx/-;T::Cre mice (Dr Moises Mallo, unpublished). This similarity in phenotypes strongly suggests that the cause of the segmental defects in the Dicer fx/-;T::Cre mouse line could be due to the dysregulation of Hox genes, which is caused by a loss of functional miRNA activity. Another reason that the

phenotype of the Dicer fx/-;T::Cre embryo skeleton could be due to the dysregulation of Hox genes is because it has very specific phenotypes: the prominent region of deformity is in the lumbar and sacral regions of the axis. Hox genes are associated with antero-posterior specification of segments within the body axis, so the mis-expression of specific Hox genes in the PSM could result in the disruption of specific vertebrae identity

Post-transcriptional regulation of Hox genes has been previously observed (Brend, 2003; Nelson et al., 1996), and this is thought to be due to microRNA (miRNA) activity. Two conserved miRNA loci exist at two positions of the Hox clusters, and target sequences are present in the 3' UTRs of neighbouring genes, indicating that miRNAs are regulating Hox mRNAs by repressing their translation (Yekta et al., 2004). *Hoxb8* mRNA is also cleaved by a miRNA (Mansfield et al., 2004). Since miRNAs are observed in embryos from an early stage, it would be logical that they could regulate these genes *in vivo*. However, although miRNA loci are found within Hox cluster and they are co-regulated with Hox genes, this does not mean that they have a role in regulating Hox genes, which remains to be explored.

Skeletal defects have long been suspected to have arisen due to the malfunction of the segmentation clock in the PSM during development, and the presence of an extra vertebra indicates that the pace of the clock may have changed as a result of a loss of miRNA function in the PSM. In light of this, I decided to investigate the gene expression profile of *Hes7*, a component of the segmentation clock, during the formation of the affected somites (E9.5 and E10.5). Due to the reliance of the clock on the short half-lives of its dynamically expressed constituents, it seemed likely that the loss of miRNA function would have some effect on the pace of the clock. However, in the whole mount embryos there were clearly distinct phases of *Hes7* expression, indicating that the segmentation clock itself has not

been affected by the loss of miRNA activity. It could be that a lack of miRNAs is causing a slight difference in the half-life of cyclic genes which our technique may not have detected. In this case, the pace of the segmentation clock would have been changed slightly, resulting in an altered number of somites being formed in the affected embryos. As we counted an extra vertebra, this would indicate that the segmentation clock was working slightly quicker than in wild type embryos. A similar phenomenon was observed recently where a Nrarp-/- embryo was shown to have a period of oscillation 5 minutes slowed as compared to wild type embryo. This mutation caused the affected mice to produce fewer vertebrae than the wild type mice (between 56-58 vertebrae in total, as opposed to 59 for Nrarp +/- and +/+ mice) (Kim et al., 2011). In order to investigate this idea further, we looked at our microarray analysis to see whether any of the cyclic genes involved in the segmentation clock were affected by the loss of miRNA activity. The only candidate clock gene which appeared to differ between the Dicer fx/-;T::Cre and wild-type datasets was *mLfng*. Although it has been reported in other labs that *Lfng* is possibly regulated by miR-125a during development (Dr. Susan Cole, The Ohio State University, personal communication), the idea that increased *Lfng* expression would result in the formation of another vertebrae does not make sense: increased *Lfng* expression and stability would likely result in the slowing of the clock pace and therefore would produce fewer vertebrae. Taken together, these observations conclude that the skeletal defects which arise in the PSM of our conditional Dicer fx/-;T::Cre mouse line are likely not caused by dysregulation of clock components, and that the mis-expression of Hox genes appears to be a more likely reason for the observed phenotypes.

Alternatively, because the affected somites are calculated to be formed between E9.5 and E10.5, the embryos that we have used in this analysis may have been the wrong stage to observe an effect, and that we should in the future examine more specific stages between E9.5 and E10.5 (i.e. E9.75, E10, E10.25), to see if the segmentation clock is affected at any of these time points when the affected somites are being generated.

The key factor in this project is the rederivastion of the Dicer fx/-;T::Cre mouse line in to our lab which is underway and which will allow us to perform a greater range of experiments than has been possible so far. Even though it appears that the segmentation clock is likely to be unaffected by the loss of miRNA activity, this could be further investigated by using a technique of a 'roller culture' *in vitro* system, where control and Dicer fx/-;T::Cre embryos are cultured from E8.5 (8-12 somites) to E9.5 (20-26 somites). These embryos would then be analysed by *in situ* hybridisation with somitic markers such as *mUncx4.1*, and following this by comparing the number of somites that are formed during this incubation period between control and Dicer fx/-;T::Cre mice. This would determine whether the pace of somitogenesis is being affected in the Dicer fx/-;T::Cre mice.

Another possibility for the formation of an extra vertebra in the Dicer fx/-;T::Cre embryos is that the patterning of the PSM may be altered as result of a loss of miRNA function, as it has been previously shown that interfering with the RA and FGF gradients causes changes to the size of the PSM, as well as affecting the forming somites. In order to investigate this I would carry out an expression analysis of markers of PSM maturation and patterning, such as: wnt3a, fgf8, and Raldh2; as well as measuring the size of the PSM and developing somites in the Dicer fx/-;T::Cre embryos and comparing them to the control embryos. What this analysis would hopefully achieve is to determine whether the absence of miRNA production

affects the patterning of the PSM, which would in turn affect the number of somites/vertebrae formed.

I will also be able to ask how is gene expression of factors regulating key steps in somitogenesis affected by the absence of miRNAs. For this I would select genes which we have previously identified by microarray analysis as being significantly affected by loss of miRNA activity and analyse their temporal and spatial expression profiles in the PSM/somites of Dicer fx/-;T::Cre mice. In order to better understand how these specific somitic malformations are generated, we will also analyse the expression of genes known to be involved in somitogenesis, including Hox genes (some were identified in the microarray as being affected by the loss of miRNA activity).

Next, I will ask how miRNA-based gene dysregulation can interfere with the process of somitogenesis. To address this question I will use the chicken embryo to perform functional studies in a two-pronged approach: Firstly, I will misexpress miRNA target genes identified above, and observe how this disrupts somite/vertebrae formation. I will then identify the specific miRNAs which regulate the 3'-UTR of target genes using cell culture luciferase assays, and use this information to develop chick embryos with inhibitors specific to the miRNA(s) to try and recapitulate the segmental abnormalities of the mutant mouse model. If I am successful in this, I will perform another microarray of these embryos to check if the same genes are affected.

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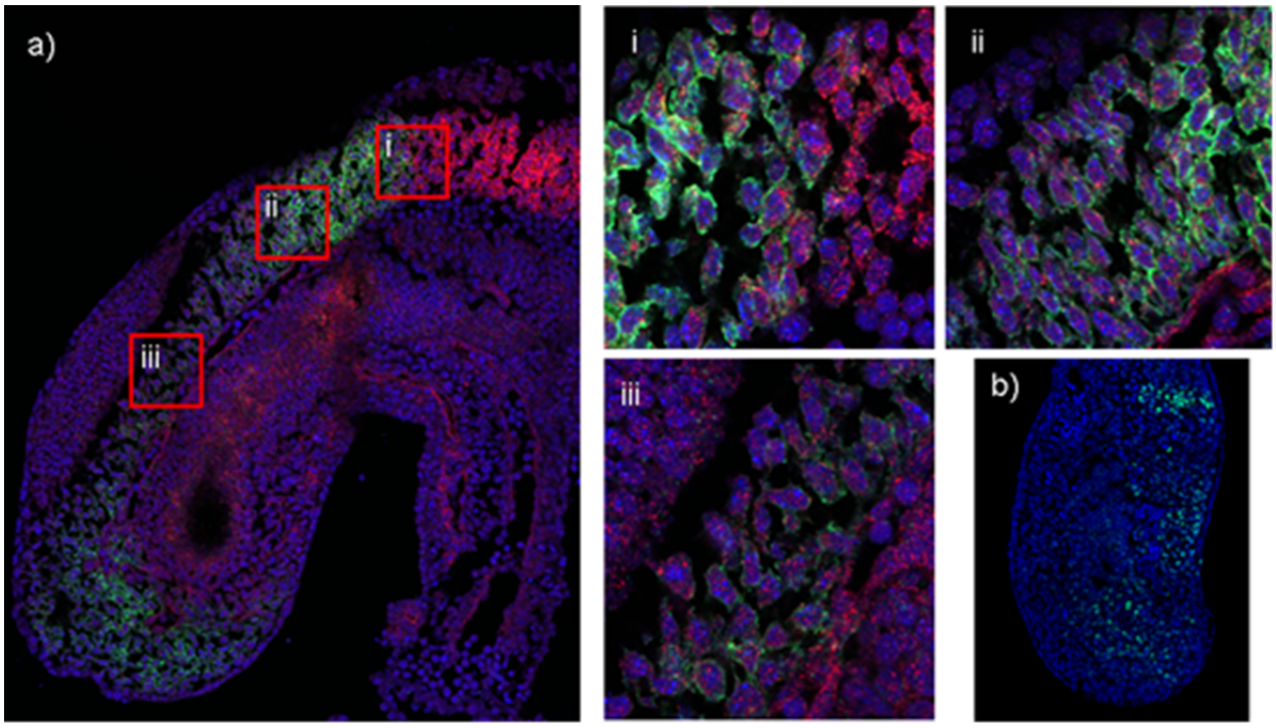
Appendix 1



a) Composit picture of a whole E10.5 mouse tail section showing both Notch1(red) and DLL1 (green) proteins via double immunohistochemistry, i)-iii): Magnified regions of different areas of the PSM; b) Phase 1 NICD immuno in an E10.5 mouse tail section.